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<b>(54) Title:</b> PROTEASE-RESISTANT THROMBOMODULIN ANALOGS  <b>(57) Abstract</b>  The present invention relates to the single-chain thrombomodulin ("TM") and analogs thereof that are not susceptible to cleavage by proteases and retain the biological activity of thrombomodulin, as well as methods of use in, for example, antithrombotic therapy. Novel proteins, nucleic acid gene sequences, pharmaceuticals and methods of inhibiting thrombotic activity are disclosed.		

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**PROTEASE-RESISTANT THROMBOMODULIN ANALOGS**

This application is a continuation-in-part of U.S. Serial No. 07/830,577, which is a continuation-in-part of U.S. Serial No. 07/568,456, filed August 15, 1990, which is a continuation-in-part of U.S. Serial No. 07/506,325, filed April 9, 1990, and the application corresponding to PCT Serial No. 90/00955, filed February 16, 1990, which was a continuation-in-part application of U.S. Serial No. 07/406,941, filed September 13, 1989, which was a continuation-in-part of U.S. Serial No. 07/345,372, filed April 28, 1989, all of whose disclosures are entirely incorporated by reference herein.

**Background of the Invention**

The present invention relates to single-chain thrombomodulin polypeptides, including analogs of thrombomodulin ("TM") that are less susceptible to cleavage by proteases. These analogs are useful in, for example, antithrombotic therapy. Novel proteins, nucleic acid gene sequences, pharmaceuticals, and methods of inhibiting thrombotic activity are disclosed.

There are many disease states that would benefit from treatment with a safe and effective anticoagulant/anti-thrombotic. The nature of these conditions varies. For example, anticoagulant therapy is useful in acute conditions such as during thrombolytic therapy in myocardial infarction or in treatment of disseminated intravascular coagulation (DIC) associated with, for example, septicemia. Anticoagulants are also useful for less acute conditions, such as chronic use in

patients that have received heart valve implants or prophylactic use in surgery patients to reduce the risk of deep venous thrombosis (DVT).

5 Thrombomodulin is a membrane protein that has demonstrated anticoagulant properties. Its physiological importance has been studied. (See, for example, N. Esmon, et al., (1982) J. Biol. Chem. 257:859-864, H. Salem, et al., (1983) J. Biol. Chem. 259:12246-12251).

10 The gene encoding native thrombomodulin has been isolated and sequenced from several species, both in its genomic form and as a cDNA clone (Jackman, R., et al., (1986) Proc. Natl. Acad. Sci. USA. 83:8834-8838 and (1987) 84:6425-6429, both of which are herein incorporated by reference). Comparisons with known  
15 proteins, such as the LDL receptor, have suggested functional domains (Wen, D., et al., (1987) Biochemistry 26:4350-4357). One study has suggested that the fifth and sixth epidermal growth factor (EGF)-like domains have the capacity to bind thrombin (Kurosawa, S., et al.,  
20 (1988) J. Biol. Chem. 263:5993-5996); another suggests that EGF-like domains 4, 5, and 6 are sufficient to act as a cofactor for thrombin-mediated protein C activating activity. (Zushi, et al., (1989) J. Biol. Chem. 264:10351-10353). Inhibition of thrombin's direct  
25 procoagulant activity (conversion of fibrinogen to fibrin) has been attributed to, in part, glycosaminoglycan substituents on the thrombomodulin molecule. (Bourin, M.C. et al., (1986) Pro. Natl. Acad. Sci. USA 83:5924-5928.) The O-linked glycosylation domain has  
30 potential sites for the addition of these types of sulfated sugars.

Thrombomodulin analogs, including soluble molecules, having various modifications are known. There are, for  
35 example, modifications to oxidation-sensitive amino acid residues in thrombomodulin which render the molecule resistant to oxidation. There are also modifications to thrombomodulin, e.g., by elimination of sulfated o-linked

The production of TM and TM analogs by recombinant techniques in heterologous cells, e.g., in cell culture, has encountered numerous problems in achieving acceptable products for use in *in vivo* applications. For example, the N-terminal end of TM is imprecisely cleaved in cells containing the recombinant gene as well as in native cells, resulting in a product that has a non-unique N-terminus. This causes, among other difficulties, a problem in providing proof of purity of the isolated polypeptide, e.g., for regulatory purposes. Glycosylation of recombinantly produced TM has also proved to be a problem, in that some of the glycosylation sites are apparently involved in maximizing biological activity, while other sites are apparently recognized as signals *in vivo* to clear the bloodstream of TM, thus reducing the circulating half-life of a TM so glycosylated. Other, less well defined problems also are known to exist which interfere with the production of a maximally useful recombinantly produced TM polypeptide.

Thus, there is a need for new compositions that exhibit the anticoagulant properties of thrombomodulin and are easily produced in large quantities, but without the problems encountered in recombinant production of TM by heterologous cells. The present invention fulfills these and other needs.

#### Summary of the Invention

This invention provides single-chain thrombomodulin (TM) substantially devoid of two-chain TM. This TM is provided by removal of two-chain TM from preparations which contain it, or by preventing cleavage of single-chain TM.

In another embodiment, this invention provides TM having a single N-terminus. This TM is provided by the absence of two-chain TM, and/or by the elimination of the natural, heterogeneous N-terminal signal sequence processing site.

carbohydrates through enzymatic removal or modification of glycosylation sites on the peptide, which decrease the inhibition of thrombin-mediated platelet aggregation and thrombin-mediated conversion of fibrinogen to fibrin, which is an important property of thrombin. These modifications are disclosed in U.S. Serial No. 07/568,456, filed August 15, 1990, which is incorporated herein by reference.

Anticoagulants currently approved for use in humans are not uniformly effective and a need exists for more efficacious compounds (See, for example, Prevention of Venous Thrombosis and Pulmonary Embolism, Consensus Development Conference Statement, NIH, 1986, 6(2):1-23).

Thrombomodulin in its native form is not suitable for anticoagulant therapy as it is membrane-bound, due to its inherent amino acid sequence, and is insoluble without detergent treatment. It is present in such small amounts (about 300 mg thrombomodulin/person) that purification from autopsy or biopsy samples is impractical.

Soluble thrombomodulin-like molecules have been detected at very low amounts in human plasma and urine. These molecules have a reduced ability to promote protein C activation, and it is possible that they have been rendered at least partially inactive, due at least in part, to oxidation. It has been suggested that these molecules are degradation products of the membrane bound molecule (Ishii, H. and Majerus, P., (1985) J. Clin. Inv. 76:2178-2181), but they are present in such low amounts that they have been difficult to characterize (~0.8 mg/adult male). Proteolytic fragments of the purified native molecule have been produced using trypsin or elastase. (See, Ishii, supra, Kurosawa, et al., (1988) J. Biol. Chem. 263:5593-5996 and Stearns, et al., (1989) J. Biol. Chem. 264:3352-3356). Some of these fragments retain the ability to promote thrombin-mediated activation of protein C *in vitro*.

In another embodiment, this invention provides TM having a single C-terminus. This TM is provided by the absence of a C-terminus which is sensitive to exocarboxypeptidase.

5 In another embodiment, this invention provides single-chain TM which can be expressed in eukaryotic cells, e.g., animal cells, vertebrate cells, insect cells, mammalian cells, human cells, etc., e.g., in CHO or CHL1 cells.

10 This invention provides methods for treating thrombotic disease by administering an effective dose of a single-chain thrombomodulin or analog thereof, typically one which is resistant to protease cleavage and which retains the biological activity of thrombomodulin.  
15 In some preferred aspects, the polypeptide composition exhibits only a single N-terminus and a single C-terminus, has at least approximately native ability to potentiate thrombin-mediated activation of protein C, and/or has a reduced ability to inactivate  
20 thrombin-mediated conversion of fibrinogen to fibrin. It is preferred in some embodiments that the analog is soluble in aqueous solution and/or has a long circulating half-life, e.g., is oxidation and/or protease resistant.

25 In other embodiments, it is preferred that the analog be modified in the sugar residues of the O-linked glycosylation domain. By modified it is meant that the O-linked glycosylation domain has an altered glycosylation pattern. This can encompass substitution, and total or partial deletion of native sugar residues. This  
30 modification can be achieved by deleting the amino acid residues that are recognized by cells as glycosylation sites, e.g., by site directed mutagenesis. Alternatively the sugars can be chemically removed, either partially or totally. In another modification the sugars can be  
35 enzymatically treated to remove sulfate substituents. In yet another modification the entire glycosylation domain can be deleted.

Some preferred analogs for use in the method will retain the capacity to potentiate the thrombin-mediated activation of protein C and/or have 80% or less of the capacity of native thrombomodulin to inactivate thrombin-mediated conversion of fibrinogen to fibrin. More specifically, these TM analogs, when standardized to have an equal activity in a standard protein C activation assay compared to native detergent-solubilized rabbit thrombomodulin, will have only 80% or less of the activity of the same amount (mass) of native thrombomodulin in a standard assay measuring thrombin-mediated conversion of fibrinogen to fibrin. One preferred analog of this invention has 50% or less of the activity of the same amount of native thrombomodulin in the fibrin assay. These capacities are measured using standard assays described herein.

This invention further provides for sterile compositions for treating thrombotic disease in mammals comprising a unit dosage of a thrombomodulin analog having one or more of the above-noted properties. The preferred analogs are as described above for various methods.

This invention further provides for methods of increasing the *in vivo* circulating half-life of a thrombomodulin analog comprising removing all or most of the sugar moieties, e.g., in the 6 EGF-like domains.

#### Brief Description of the Figures

Figure 1 shows an elution and activity profile of a soluble TM<sub>0</sub> (Sf9) preparation resolved on a Mono Q column.

Figure 2 shows SDS-PAGE analysis of samples from the column profile shown in Figure 1.

Figure 3 shows gel electrophoresis profiles of samples run under reducing conditions.

Figure 4 shows a Western blot of a gel electrophoresis profile.



Figure 5 shows a double reciprocal plot of TM binding to thrombin.

Figure 6 shows SDS-PAGE of samples from an anhydrotrypsin column, reduced in sample buffer in 10%  $\beta$ -mercaptoethanol and loaded on an 8% SDS-PAGE gel.  $TM_{LEO}(CHO)M_{388}L$  lot #102491A (load or starting material), clipped  $TM_{LEO}(CHO)M_{388}L$  (bound or pool), intact  $TM_{LEO}(CHO)M_{388}L$  (flow through), and positions of molecular weight markers (kDa) are indicated.

Figure 7 shows a gel scan of the SDS-PAGE gel of Fig. 6. The gel was scanned on a Molecular Devices Computing Densitometer and analyzed using the program ImageQuant provided with the instrument.  $TM_{LEO}(CHO)M_{388}L$  lot #102491A (load or starting material) has a shoulder indicated by the arrow which is the clipped  $TM_{LEO}(CHO)M_{388}L$ . This shoulder is removed by the anhydrotrypsin column from intact  $TM_{LEO}(CHO)M_{388}L$  (flow through). Clipped  $TM_{LEO}(CHO)M_{388}L$  is eluted in pure form from the column.

#### Detailed Description of the Invention

In accordance with the present invention, new TM analogs, methods, and compositions are provided which can treat thrombotic disease. They are based on a single-chain thrombomodulin (TM) or thrombomodulin analog which is resistant to protease cleavage. In addition, other modifications can be introduced which result in other improved properties of an antithrombotically effective pharmaceutical, e.g., wherein the TM exhibits a reduced capacity to inhibit the direct procoagulant activities of thrombin or exhibits other properties such as thrombin-mediated conversion of fibrinogen to fibrin, oxidation resistance, glycosylation resistance, increased in vivo half-life, etc. Pharmacologists prefer drugs which contain a single therapeutically effective homogeneous composition. Such drugs are preferred because they are less likely to induce undesired side effects than drugs containing multiple species, including species containing

undefined biological effectiveness. This invention has the advantage of containing a more biologically pure species, often also being chemically pure, which does not have the disadvantages of prior art products.

5       The prior art thrombomodulin compositions, particularly those produced by recombinant techniques in heterologous cells, have been studied extensively by the present inventors in order to determine parameters for the structure of the polypeptide which provide optimal  
10       pharmaceutical utility. In the course of these investigations, it was found that, surprisingly, thrombomodulin preparations previously thought to contain pure, single-chain polypeptides were, in fact, heavily contaminated with thrombomodulin polypeptides having an  
15       internal peptide cleavage, but wherein the polypeptide continued to copurify with single-chain forms of TM, because the cysteine bonding between the separate chains caused the two-chain form of the TM to behave similarly to a single-chain form under purification conditions.  
20       This would apply both to full-length native TM, as well as to soluble TM molecules.

      This previously unappreciated problem was discovered, in part, by careful analysis of these preparations, which revealed that the recombinantly  
25       produced thrombomodulin demonstrated two-phase non-linear binding properties. Double-reciprocal plot analysis indicated the presence of two species with different binding affinities in the composition. This led to further investigations as to the nature of the different  
30       species. Careful analysis of TM preparations using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) run under reducing, non-reducing, or native conditions and employing sensitive detection techniques, e.g., silver or immunological staining, led to the  
35       discovery that there was a second immunoreactive band having an apparent molecular weight of about 80 kilodaltons, in addition to the expected 94 kilodalton

band of particular soluble TM analogs, on such gels. This composition was then shown to have a second N-terminal amino acid sequence consistent with the presence of an internal N-terminus. This is likely the result of a protease cleavage, at a location within the TM sequence, indicating that the apparently single-chain form of TM was contaminated with a cleaved two-chain species. This species is cleaved near or within the thrombin binding site of the TM. Thus, while the physical characteristics of the two-chain form were so similar to the single-chain form as to result in copurification, in fact the biological activity, e.g., the binding properties, of the mixture appears to be affected by this previously unrealized distinct species. The present invention provides thrombomodulin analog compositions substantially free of such two-chain contaminants.

In another aspect of this invention, there are provided other improved compositions of thrombomodulin. For example, it has also been discovered that TM analogs, especially those produced by heterologous cells under recombinant conditions have an N-terminus produced by signal sequence processing of the amino acid sequences upstream of the mature N-terminus. In fact, the product exhibits heterogeneity at the amino terminus. There are two processing sites, one which provides the native N-terminal amino acid 1, and a second site which, when used, produces a cleavage product containing two more amino acids at the N-terminus, e.g., starting at amino acid -2. By modification of the DNA sequence encoding the N-terminus of the TM polypeptide, the signal sequence processing site can be modified to provide a single N-terminal processing site, and therefore a single N-terminus for the resultant TM analogs. Thus, a TM analog which deletes the first three amino acids of the native molecule provides a signal sequence processing

site which is unique, thereby producing a polypeptide composition with a single N-terminal sequence.

Still further, the elimination of internal cleavages to produce a single-chain TM analog results in a composition having a homogeneous carboxy terminus. Constructs which are resistant to protease or other proteolytic cleavage are made so as to provide single C-termini as well. This can be accomplished by removal or modification of protease cleavage sites. For example, by deleting a portion of the C-terminal region of the polypeptide which is not critical to biological function in pharmaceutical applications, a polypeptide can be provided which terminates in a -Pro-Pro sequence at amino acids 489-490, which sequence is especially resistant to exocarboxypeptidase activity.

In another embodiment, this invention also provides for methods of increasing the *in vivo* half-life of TM analogs by modifying or deleting particular native glycosylation patterns, or removing protease-sensitive sequences. Our early studies have indicated that the proteolysis-resistant form of soluble TM has a longer circulating half-life in an animal. Thus, it is likely that a physiological mechanism which contributes to TM inactivation is proteolysis at, or near, the identified region of the protein. By modifying this segment of the sequence to avoid inactivation, a much more effective therapeutic agent is produced. Increased half-life is advantageous for TM therapy because it permits administration of lesser amounts of TM to achieve equivalent pharmacological effect compared to the native drug. A biological half-life which is at least greater than a few minutes provides for a more predictable therapeutic regimen.

In addition, these soluble thrombomodulin analogs can be produced economically and are easily purified and administered. A variety of therapeutic uses are anticipated, particularly with respect to anticoagulant and/or

antithrombotic therapies. In order to fully appreciate the invention, the following detailed description is set forth.

I. Biological Activity of Thrombomodulin

5       The underlying pathology of thrombotic disorders is that a clot forms in response to a stimulus such as, for example, a damaged vessel wall. This stimulus triggers the coagulation cascade and thus generates thrombin which has the ability to convert fibrinogen to fibrin, the  
10       matrix of the clot. Thrombomodulin is an endothelial cell membrane protein that acts as a receptor for thrombin. In humans it is distributed on the endothelium of the blood vessels and lymphatics of all organs except the central nervous system. Thrombin has the ability to  
15       bind reversibly to thrombomodulin. When bound to thrombomodulin, thrombin is converted from a procoagulant enzyme to an anticoagulant enzyme. The thrombin/-thrombomodulin complex inhibits the coagulation cascade in at least two distinct ways. First, thrombin's binding  
20       to thrombomodulin potentiates thrombin-mediated activation of protein C. Activated protein C inactivates other procoagulant components of the coagulation cascade, such as Factors Va and VIIIa, which in turn inhibits the conversion of more prothrombin to thrombin. Thrombin-mediated activation of protein C is greatly enhanced when  
25       thrombin is bound to thrombomodulin, i.e., the rate of protein C activation increases at least 1000-fold. Secondly, binding to thrombomodulin has direct anti-coagulant effects such as the inhibition of thrombin-mediated conversion of fibrinogen to fibrin and thrombin-mediated activation and aggregation of platelets.  
30       Although normally an integral component of the endothelial cell membrane, thrombomodulin can be released from the membrane in the presence of sufficient detergent and retains the ability to bind to thrombin when in  
35       solution.

The preferred thrombomodulin analogs of this invention will protect against thrombus formation when administered systemically because they will inhibit the generation of thrombin without disturbing other coagulation parameters, e.g., the activation and aggregation of platelets. Thus the use of soluble thrombomodulin analogs will be effective at preventing thrombus formation, yet is safer than native thrombomodulin and other antithrombotics known in the art.

Diseases in which thrombus formation plays a significant etiological role include myocardial infarction, disseminated intravascular coagulation, deep vein thrombosis, pulmonary embolism, septic shock, acute respiratory distress syndrome, unstable angina, and other arterial or venous occlusive conditions. The thrombomodulin analogs of this invention are useful in all of these, as well as in other diseases in which thrombus formation is pathological. By useful it is meant that the compounds are useful for treatment, either to prevent the disease or to prevent its progression to a more severe state. The compounds of this invention also provide a safe and effective anticoagulant, for example, in patients receiving bioprostheses, such as heart valves. These compounds may replace heparin and warfarin in the treatment of, for example, pulmonary embolism or acute myocardial infarction.

In particular these compounds would find a role in the prevention of deep vein thrombosis (DVT), for instance after surgery. The formation of blood clots in the leg is itself a non-fatal condition but is very closely tied to the development of pulmonary embolism (PE), which is difficult to diagnose and can be fatal. Despite the investigation and clinical use of several prophylactic regimens, DVT and the resulting PE remain a significant problem in many patient populations and particularly in patients undergoing orthopedic surgery. Existing prophylactic treatments such as heparin,

warfarin, and dextran typically reduce the incidence of DVT in orthopedic surgery patients from more than 50% in patients at risk receiving no prophylaxis to 25-30% among treated patients. There are serious side effects, primarily bleeding complications. Currently, daily laboratory tests and adjustments in dosage are required to minimize bleeding episodes while retaining some efficacy. Based on the shortcomings of existing prophylactics, an antithrombotic which is effective at preventing DVT without predisposing the patient to bleeding could make a significant impact on patient recovery and well-being.

Angioplasty is a procedure frequently used for restoring patency in occluded arteries. Although patency may be restored, it is inherent in an angioplasty procedure that the endothelial lining of the artery is severely damaged, and blood clots frequently begin to form. Soluble thrombomodulin analogs administered in conjunction with angioplasty will prevent this deleterious side effect.

Many acute thrombotic and embolic diseases are currently treated with fibrinolytic therapy in order to remove the thrombus. The condition that has been most investigated is acute myocardial infarction (heart attack). Agents currently in use for treating acute myocardial infarction include streptokinase, tissue plasminogen activator and urokinase. Use of these agents can lead to serious bleeding complications. Patients who have had a thrombus removed by fibrinolytic therapy and in whom the blood flow has been restored frequently reocclude the affected vessel, i.e., a clot reforms. Attempts have been made to prevent the reocclusions by increasing the dose or time of treatment with a thrombolytic agent, but the incidence of bleeding then increases. Thus the therapeutic index for these drugs is narrow.

The use of thrombomodulin analogs provides protection against reocclusion, in part because its action is local, i.e., where thrombin is being generated or being released from a clot. Therefore, when used in combination with a thrombolytic agent whose dose can then be decreased, the risk of bleeding can be substantially reduced.

Administration of single-chain thrombomodulin or TM analogs can be accomplished by a bolus intravenous injection, by a constant intravenous infusion, or by a combination of both routes. Also, soluble thrombomodulin mixed with appropriate excipients may be taken into the circulation from an intramuscular site. Systemic treatment with thrombomodulin analogs can be monitored by determining the activated partial thromboplastin time (APTT) on serial samples of blood taken from the patient. The coagulation time observed in this assay is prolonged when a sufficient level of thrombomodulin is achieved in the circulation. However, this is a systemic measurement of efficacy, and the inventors have discovered that an effective dose of soluble TM analog does not necessarily affect the APTT. As used herein, a therapeutically effective dose is defined as that level of TM analog sufficient to prevent formation of pathological clots. Dosing levels and regimens can be routinely adjusted by one of ordinary skill in the art so that an adequate concentration of thrombomodulin is maintained as measured by, for example, the activated partial thromboplastin clotting time (APTT), the thrombin clotting time (TCT), or conversion of protein C to activated protein C (APC) assays.

Several methods are known for the detection and monitoring of thrombotic disease. Deep venous thrombosis can be detected, for example, by contrast venography, (Kerrigan, G.N.W., et al., (1974) British Journal of Hematology 26:469), Doppler ultrasound (Barnes, R.W. (1982) Surgery Clinics in North America 62:489-500), "I-



labeled fibrinogen uptake scanning (Kakkar, V.V., et al., (1972) Archives of Surgery 104:156; Kakkar, V.V., et al., (1970) Lancet i:540-542), impedance plethysmography (Bynum, L.J. et al., (1978) Annals of Internal Medicine 89:162), and thromboscintiscan (Ennis, J.T. and Elmes, R.J. (1977) Radiology 125:441). These methods are useful to monitor the efficacy of the methods and compositions described herein.

## II. TM analogs

A DNA sequence encoding the full-length native human thrombomodulin protein has been isolated (European Patent Application No. 88870079.6, which is incorporated herein by reference). The cDNA sequence encodes a 60.3 kDa protein of 575 amino acids, which includes a signal sequence of about 18 amino acids.

The sequences for bovine, mouse and human thrombomodulin exhibit a high degree of homology with one another. By analogy with other proteins, the structure of thrombomodulin can be presumptively divided into domains. The term "domain" refers to a discrete amino acid sequence that can be associated with a particular function or characteristic. Typically, a domain exhibits a characteristic tertiary structural unit. The full-length thrombomodulin gene encodes a precursor peptide containing the following domains:

<u>Approximate Amino Acid Position</u>	<u>Domain</u>
-18--1	Signal sequence
1-226	N-terminal (lectin) domain (L)
227-462	6 EGF-like domains (E)
463-497	O-linked Glycosylation (O)
498-521	Stop Transfer Sequence (transmembrane domain)
522-557	Cytoplasmic domain

See C. S. Yost et al., (1983) *Cell*, 34:759-766 and D. Wen et al., (1987) *Biochemistry*, 26:4350-4357, both incorporated herein by reference.

5 In the nomenclature used here, the subscript refers to the domains contained in the TM analog: L = the lectin domain, E = the 6 EGF-like domains, O = the O-linked glycosylation domain, M = the transmembrane domain, and C = the cytoplasmic domain. Thus, TM analog 6h/227-462 corresponds to a TM<sub>E</sub> analog, TM<sub>E</sub>(Sf9) indicates 10 that it is expressed in insect cells, TM<sub>LEO</sub>(CHO) indicates that it is expressed in CHO cells, and TMD123 (Zushi, M., Gomi, K., Yamamoto, S., Maruyama, I., Hayashi, T., and Suzuki, K. (1989) *J. Biol. Chem.* 264, 10351-10353) and TMD1 (Parkinson, J.F., Grinnell, B.W., Moore, R.E., 15 Hoskins, J., Vlahos, C.J., and Bang, N.U. (1990) *J. Biol. Chem.* 265, 12602-12610) correspond to TM<sub>LEO</sub> analogs.

Particularly preferred single-chain TM analog compositions are those that have one or more of the following characteristics:

- 20 (i) they exhibit protease resistance,
- (ii) they have homogeneous N- or C-termini,
- (iii) they have been post-translationally modified, e.g., by glycosylation of at least some of the glycosylation sites of native thrombomodulin,
- 25 (iv) they have linear double-reciprocal thrombin binding properties,
- (v) they are soluble in aqueous solution in relatively low amounts of detergents and typically lack a stop transfer (transmembrane) sequence,
- 30 (vi) they retain activity after exposure to oxidants,
- (vii) when bound to thrombin, they potentiate the thrombin-mediated activation of protein C but have a reduced ability to inhibit the direct pro-coagulant activities of thrombin such as 35 the conversion of fibrinogen to fibrin or the activation and aggregation of platelets.

Assays for the last two characteristics can be run on an automatic coagulation timer according to the manufacturer's specifications; Medical Laboratory Automation Inc. distributed by American Scientific Products, McGaw Park, Illinois. (See also H. H. Salem et al., (1984) J. Biol. Chem., 259:12246-12251, which is incorporated herein by reference). In comparison to native thrombomodulin, preferred TM analogs have been modified to embrace the 6 epidermal growth factor [EGF]-like domains and may also contain the O-linked glycosylation and/or lectin domains.

In a preferred embodiment, soluble TM analogs are oxidation resistant. This refers to analogs that retain activity after exposure to oxidants. Such analogs are described in detail in co-pending co-assigned U.S. Application Serial No. 07/506,325, filed April 9, 1990, incorporated herein by reference.

For purposes of the present invention, the following terms are defined:

"Protease site" as used herein refers to an amino acid or series of amino acids in a TM polypeptide which define a recognition, binding, cleavage, or other site susceptible to the activity of a protease. For example, when one or more amino acid residues encompassed by this site are substituted by another amino acid residue(s) or are deleted, the protease is no longer able to cleave the TM at that site. This term also encompasses regions of the TM molecule which are inherently susceptible to proteases, e.g., by being conformationally exposed and available to a protease activity.

"Protease cleavage site" as used herein refers to the precise location at which a protease cleaves the TM polypeptide analog.

"Single N-terminus" and "single C-terminus" are used herein to have their literal meanings which functionally refer to the property of the composition, e.g., wherein, upon conventional sequential amino acid sequence

analysis, each degradation cycle results in the removal of an amino acid residue which is essentially devoid of a different amino acid residue. Thus, after several cycles, e.g., 10 cycles, of stepwise removal of the N-terminal amino acids, essentially only one amino acid is recovered at each cycle. In particular, no more heterogeneity in sequence is detected than would be statistically expected from a completely pure single-chain polypeptide according to the analytic procedure used.

"Single-chain TM" refers to a composition of TM which contains substantially all uncleaved peptide chains. The polypeptides in a single-chain composition need not all exhibit identical amino or carboxy terminal ends.

"Two-chain TM" refers to a composition containing physically detectable amounts, typically in excess of about .5-3%, of polypeptide which has a broken peptide bond.

"Devoid of two-chain thrombomodulin" as used herein means that the composition comprises essentially all single-chain TM. Typically, the amount of two-chain TM is less than about 25% by molar amount, more typically less than 15%, preferably less than about 10%, more preferably less than 5%, and in particularly preferred embodiments, less than 3%. Alternatively, the amount of two-chain TM in a composition is less than that which would cause a significant decrease in the specific activity of pure single-chain TM, e.g., the amount of two-chain TM is less than the amount which alters a linear double reciprocal plot defined herein. Thus, two-chain TM molecules are present in the form of a polypeptide chain having at least one scission which results in the production of additional N- and/or C-termini.

"Substantially retains the biological activity of native thrombomodulin" and similar terms, as used herein,

means that the thrombomodulin shares biological activities with a native membrane bound TM molecule. Generally, the activity in units per milligram of protein is at least about 50%, ordinarily 75%, typically 85%, more typically 95%, preferably 100% and more preferably over 100% of the activity of native thrombomodulin. This biological activity can be that of thrombin-mediated activation of protein C (APC), of activated partial thromboplastin clotting time (APTT), of thrombin clotting time (TCT), or of any of TM's biological, preferably therapeutic, activities. The native standard of comparison is a full-length membrane bound version of TM, but in many cases, a soluble TM comprising the lectin/EGF/O-linked domain (TM<sub>LEO</sub>) can be used as a more convenient standard.

"Glycosylation sites" refer to amino acid residues which are recognized by a eukaryotic cell as locations for the attachment of sugar residues. The amino acids where sugars are attached are typically Asn (for N-linked sugars), threonine or serine (for O-linked sugars) residues. The specific site of attachment is typically signaled by a sequence of amino acids, e.g., Asn-X-(Thr or Ser) for most N-linked attachment and (Thr or Ser)-X-X-Pro for most O-linked attachment, where X is any amino acid. The recognition sequence for glycosaminoglycans (a specific type of sulphated sugar) is generally Ser-Gly-X-Gly, but can also be X-Ser-Gly-X-Val. The terms N-linked and O-linked refer to the chemical group that serves as the attachment site between the sugar moiety and the amino acid residue. N-linked sugars are attached through an amido group; O-linked sugars are attached through an hydroxyl group.

"In vivo circulating half-life" refers to the average time it takes an administered plasma activity in a mammal to decrease by one half.

"Native thrombomodulin" refers to the full length protein as it occurs in nature. When biological

activities are described with reference to the native TM, the term embraces a detergent solubilized aqueous form. Often, in the context of comparison to an activity, a transfected soluble polypeptide may exhibit substantially identical properties.

"O-linked glycosylation domain" refers to the sequence of amino acids numbered from 463 through 485 of the native thrombomodulin sequence as depicted in Table 1.

"Oxidation resistant analogs" refers to analogs of thrombomodulin which are able to maintain a substantial amount of biological activity after exposure to an oxidizing agent such as oxygen radicals, Chloramine T, hydrogen peroxide, or activated neutrophils.

"Pharmaceutical excipients" refers to non-toxic, medically-acceptable materials which are used to complete a medical therapeutic. These materials can be inert, such as water and salt, or biologically active, such as an antibiotic or analgesic.

"Reduced ability" refers to a statistically meaningful lowering of a biological property. The property is unlimited and the measurement or quantification of the property is by standard means.

"Sugar residues" refers to hexose and pentose carbohydrates including glucosamines and other carbohydrate derivatives and moieties which are covalently linked to a protein.

"Sulfate substituents" are sulfur-containing substituents on pentose or hexose sugars.

"Thrombin-mediated conversion of fibrinogen to fibrin" refers to the enzymatic activity by which thrombin cleaves the precursor protein fibrinogen to make fibrin monomer, which subsequently polymerizes to form a blood clot.

"Thrombotic disease" refers to a pathogenic condition in a mammal characterized by the formation of

one or more thrombi that are or can be detrimental to the health of the mammal.

"Thrombomodulin analogs" are peptides which substantially retain the biological activity of natural TM, as discussed above, and which have a molecular structure different from that of a natural version TM. For example, the term refers to proteins having an amino acid sequence identical or homologous with that of native thrombomodulin, to insoluble and soluble thrombomodulin peptides or fragments, and to oxidation resistant TM species, all having thrombomodulin-like activity. These compounds also include derivatives and molecules comprising amino acid changes which do not significantly decrease the protein C activation cofactor properties of the protein when compared with native TM.

"Transfer vector" refers to a vector cotransfected into another cell, e.g., an insect cell, with, e.g., a wild-type baculovirus. The transfer vector is constructed in such a way as to encourage a recombination between a viral, e.g., the baculovirus, genome and the transfer vector, e.g., replacing the baculovirus polyhedron gene with a heterologous target gene. Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome.

As used herein, a "soluble TM analog" is a TM analog which is soluble in an aqueous solution, and typically can be secreted by a cell. For pharmacological administration, the soluble TM analog or an insoluble analog comprising the native cytoplasmic domain or analog, may optionally be combined with phospholipid vesicles, detergents, or other similar compounds well known to those skilled in the art of pharmacological formulation. The preferred TM analogs of the present invention are soluble in the blood stream, making the analogs useful in various anticoagulant and other therapies. These modifi-

cations typically do not significantly affect many activities relative to native thrombomodulin, e.g., affinity for thrombin or activity in protein C activation.

Two preferred analogs encompass the 6 EGF-like domains and are 4t/227-462 where the analog has the last four residues of the human tissue plasminogen activator signal peptide and 6h/227-462 where the 6h represents the last six residues of the hypodermin A signal sequence. More preferred are these analogs rendered oxidation resistant by substitution of the methionine at position 388 with leucine.

Another preferred embodiment is an analog corresponding to amino acids 3-490, with modifications to Met388, Arg456, His457, Ser474, and deletions at the N- and C-termini. This embodiment is particularly useful when expressing the gene in eukaryotic cells, e.g., in animal cells, vertebrate cells, insect cells, mammalian cells, human cells, etc., in particular, CHO and CHL1 cells.

#### 20 A. General Methods For Making TM Analogs

This invention embraces molecular genetic manipulations that can be achieved in a variety of known ways. The recombinant cells, plasmids, and DNA sequences of the present invention provide a means to produce pharmaceutically useful compounds wherein the compound, secreted from recombinant cells, is a soluble derivative of thrombomodulin.

Generally, the definitions of nomenclature and descriptions of general laboratory procedures used in this application can be found in J. Sambrook et al., Molecular Cloning, A Laboratory Manual, (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. The manual is hereinafter referred to as Sambrook and is hereby incorporated by reference. In addition, Ausubel et al., eds., Current Protocols in Molecular Biology, (1987 and periodic updates) Greene Publishing Associates,



Wiley-Interscience, New York, discloses methods useful in the present application.

All enzymes are used according to the manufacturer's instructions.

5       Oligonucleotides that are not commercially available can be chemically synthesized according to the solid phase phosphoramidite triester method first described by S.L. Beaucage and M.H. Caruthers, (1981) Tetrahedron Letts., 22(20):1859-1862 using an automated synthesizer, 10 as described in D.R. Needham-VanDevanter et al., (1984) Nucleic Acids Res., 12:6159-6168. Purification of oligonucleotides was by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson, J.D., and Regnier, F.E. (1983) J. Chrom., 15 255:137-149. Nucleotide sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis or from published DNA sequences.

20       The sequence of the cloned genes and synthetic oligonucleotides can be verified using the chemical degradation method of Maxam, A.M., et al., (1980) Methods in Enzymology, 65:499-560, or similar methods. The sequence can be confirmed after the assembly of the oligonucleotide fragments into the double-stranded DNA 25 sequence using the method of Maxam and Gilbert, supra, or the chain termination method for sequencing double-stranded templates of Wallace, R.B., et al., (1981) Gene, 16:21-26. Southern Blot hybridization techniques were carried out according to Southern et al., (1975) J. Mol. Biol., 98:503. 30

35       Embodiments of this invention often involve the creation of novel peptides and genes by *in vitro* mutagenesis. Target genes are isolated in intermediate vectors and cloned for amplification in prokaryotes such as *E. coli*, *Bacillus*, or *Streptomyces*. Most preferred is *E. coli* because that organism is easy to culture and more fully understood than other species of prokaryotes. The

Sambrook manual contains methodology sufficient to conduct all subsequently described clonings in *E. coli*. Strain MH-1 is preferred unless otherwise stated. All *E. coli* strains are grown on Luria broth (LB) with glucose, or M9 medium supplemented with glucose and acid-hydrolyzed casein amino acids. Strains with resistance to antibiotics were maintained at the drug concentrations described in Sambrook. Transformations were performed according to the method described by Morrison, D.A. (1977) J. Bact., 132:349-351 or by Clark-Curtiss, J.E., and Curtiss, R. (1983) Methods in Enzymology, 101:347-362, Eds. R. Wu et al., Academic Press, New York. Representative vectors include pBR322 and the pUC series which are available from commercial sources.

#### 15           B.   Gene Synthesis

The gene encoding native thrombomodulin has been isolated and sequenced from several species, both in its genomic form and as a cDNA (Jackman, R., et al., (1986) Proc. Natl. Acad. Sci. USA. 83:8834-8838 and (1987) 84:6425-6429, both of which are herein incorporated by reference).

Publication of the full length DNA sequence encoding human thrombomodulin and thrombin facilitates the preparation of genes and is used as a starting point to construct DNA sequences encoding TM peptides. See, e.g., Genbank Register c/o IntelliGenetics, Inc., Mountain View, California. The peptides of the present invention are preferably soluble derivatives which lack the stop transfer sequence of TM in addition to having internal amino acid substitutions. Furthermore, these analogs are secreted from eukaryotic cells which have been transfected or transformed with plasmids containing genes which encode these polypeptides. Methods for making modifications, such as amino acid substitutions, deletions, or the addition of signal sequences to cloned

genes are known. Specific methods used herein are described below.

The full-length gene for thrombomodulin can be prepared by several methods. Human genomic libraries are commercially available. Oligonucleotide probes, specific to these genes, can be synthesized using the published gene sequence. Methods for screening genomic libraries with oligonucleotide probes are known. The publication of the gene sequence for thrombomodulin demonstrates that there are no introns within the coding region. Thus a genomic clone provides the necessary starting material to construct an expression plasmid for thrombomodulin using known methods.

A thrombomodulin encoding DNA fragment can be retrieved by taking advantage of restriction endonuclease sites which have been identified in regions which flank or are internal to the gene. (Jackman, R.W., et al., (1987) Proc. Natl. Acad. Sci. USA., 84:6425-6429).

Alternatively, the full length genes can also be obtained from a cDNA library. For example, messenger RNA prepared from endothelial cells provides suitable starting material for the preparation of cDNA. A cDNA molecule containing the gene encoding thrombomodulin is identified as described above. Methods for making cDNA libraries are well known (See Sambrook, supra).

Genes encoding TM peptides may be made from wild-type TM genes first constructed using the gene encoding full length thrombomodulin. A preferred method for producing wild-type TM peptide genes for subsequent mutation combines the use of synthetic oligonucleotide primers with polymerase extension on a mRNA or DNA template. This polymerase chain reaction (PCR) method amplifies the desired nucleotide sequence. U.S. Patents 4,683,195 and 4,683,202 describe this method.

Restriction endonuclease sites can be incorporated into the primers. Genes amplified by the PCR reaction can be purified from agarose gels and cloned into an appropriate

vector. Alterations in the natural gene sequence can be introduced by the techniques of *in vitro* mutagenesis or by use of the polymerase chain reaction with primers that have been designed to incorporate appropriate mutations.

5 See Innis, M. et al., eds. (1990), PCR Protocols: A Guide to Methods and Applications, Academic Press.

The TM peptides described herein are typically secreted when expressed in eukaryotic cell culture. Secretion may be obtained by the use of the native signal  
10 sequence of the thrombomodulin gene. Alternatively, genes encoding the TM peptides of the present invention may be ligated in proper reading frame to a signal sequence other than that corresponding to the native thrombomodulin gene. For example, the signal sequence of  
15 t-PA, (see WO 89/00605 incorporated herein by reference) or of hypodermin A or B (see EP 326,419 which is incorporated hereby by reference) can be linked to the polypeptide (See Table 2). In a preferred embodiment of the present invention, use is made of the signal sequence  
20 of t-PA which contains the second intron of the human t-PA gene. The inclusion of the intron enhances the expression level of the adjacent structural gene.

With some analogs of this invention, those portions of the gene encoding the stop transfer and cytoplasmic  
25 domains of the carboxyl terminal region of the native thrombomodulin gene are deleted. Therefore, it is necessary to add a stop codon so that translation will be terminated at the desired position. Alternatively, a stop codon can be provided by the desired expression  
30 plasmid. Additionally, a polyadenylation sequence can be utilized to ensure proper processing of the mRNA in eukaryotic cells encoding the TM analog. Also, it may be useful to provide an initiation codon, if one is not present, for expression of the TM peptides. Such  
35 sequences may be provided from the native gene or by the expression plasmid.

Cloning vectors suitable for replication and integration in prokaryotes or eukaryotes and containing transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of TM peptides are described herein. The vectors are comprised of expression cassettes containing at least one independent terminator sequence, sequences permitting replication of the plasmid in both eukaryotes and prokaryotes, i.e., shuttle vectors, and selection markers for both prokaryotic and eukaryotic systems.

C: Expression of TM Peptides in Prokaryotic Cells

In addition to the use of cloning methods in *E. coli* for amplification of cloned nucleic acid sequences it may be desirable to express TM analogs in prokaryotes. As discussed in greater detail below, the carbohydrate moieties of the mature protein are not essential for activity as a cofactor for the activation of protein C but do have an effect on the direct anticoagulant properties of the TM analogs as well as the molecule's half-life in circulation. Expression of thrombomodulin analogs in *E. coli* has provided a useful tool for analysis of this issue. It is possible to recover a therapeutically functional protein from *E. coli* transformed with an expression plasmid encoding a soluble TM analog.

Methods for the expression of cloned genes in bacteria are well known. To obtain high level expression of a cloned gene in a prokaryotic system, it is often essential to construct expression vectors which contain, at the minimum, a strong promoter to direct mRNA transcription termination. Examples of regulatory regions suitable for this purpose are the promoter and operator region of the *E. coli*  $\beta$ -galactosidase gene, the *E. coli* tryptophan biosynthetic pathway, or the leftward promoter from the phage lambda. The inclusion of selection markers in DNA vectors transformed in *E. coli* are useful.

Examples of such markers include the genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

See Sambrook, supra, for details concerning selection markers and promoters for use in *E. coli*. In one described embodiment of this invention, pUC19 is used as a vector for the subcloning and amplification of desired gene sequences.

D. Expression of TM Peptides in Eukaryotic Cells

It is expected that those of skill in the art are knowledgeable in the expression systems chosen for expression of the desired TM peptides and no attempt to describe in detail the various methods known for the expression of proteins in eukaryotes will be made.

The DNA sequence encoding a soluble TM analog can be ligated to various expression vectors for use in transforming host cell cultures. The vectors typically contain marker genes and gene sequences to initiate transcription and translation of the heterologous gene.

The vectors preferably contain a marker gene to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase, metallothionein, hygromycin, or neomycin phosphotransferase. The nuclear polyhedral viral protein from *Autographa californica* is useful to screen transfected insect cell lines from *Spodoptera frugiperda* and *Bombyx mori* to identify recombinants. For yeast, Leu-2, Ura-3, Trp-1, and His-3 are known selectable markers (Gene (1979) 8:17-24). There are numerous other markers, both known and unknown, which embody the above scientific principles, all of which would be useful as markers to detect those eukaryotic cells transfected with the vectors embraced by this invention.

Of the higher eukaryotic cell systems useful for the expression of TM analogs, there are numerous cell systems to select from. Illustrative examples of mammalian cell

lines include RPMI 7932, VERO, and HeLa cells, Chinese hamster ovary (CHO) cell lines, WI38, BHK, COS-7, C127, or MDCK cell lines. A preferred mammalian cell line is CHL-1 or CHO. When CHL-1 is used, hygromycin is included as a eukaryotic selection marker. CHL-1 cells are derived from RPMI 7932 melanoma cells, a readily available human cell line. The CHL-1 cell line has been deposited with the ATCC according to the conditions of the Budapest Treaty and has been assigned #CRL 9446, deposited June 18, 1987. Cells suitable for use in this invention are commercially available from the American Type Culture Collection. Illustrative insect cell lines include *Spodoptera frugiperda* (fall Armyworm) and *Bombyx mori* (silkworm).

As indicated above, the expression vector, e.g., plasmid, which is used to transform the host cell, preferably contains gene sequences to initiate the transcription, and sequences to control the translation of the TM peptide gene sequence. These sequences are referred to as expression control sequences. When the host cell is of insect or mammalian origin, illustrative expression control sequences include but are not limited to the following: the retroviral long terminal repeat promoters ((1982) Nature, 297:479-483), SV40 promoter ((1983) Science, 222:524-527, thymidine kinase promoter (Banerji, J., et al., (1982) Cell, 27:299-308), or the beta-globin promoter (Luciw, P.A., et al., (1983) Cell, 33:705-716). The recipient vector nucleic acid containing the expression control sequences is cleaved using restriction enzymes and adjusted in size as necessary or desirable. This segment is ligated to a DNA sequence encoding a TM peptide by means well known in the art.

When higher animal host cells are employed, polyadenylation or transcription termination sequences normally need to be incorporated into the vector. An example of a polyadenylation sequence is the

polyadenylation sequence from SV40, which may also function as a transcription terminator.

Genes incorporated into the appropriate vectors can be used to direct synthesis of proteins in either transient expression systems or in stable clones. In the former case yields are low, but the experiments are quick. In the latter case it takes more time to isolate high producing clones. Different vectors may be used for the two different types of experiments. In particular, in the case of transient expression, sequences may be included within the plasmid that allow the plasmid to replicate to a high copy number within the cell. These sequences may be derived from a virus such as SV40 (e.g., Doyle, C. et al., (1985) J. Cell Biol., 100:704-714) or from chromosomal replicating sequences such as murine autonomous replicating sequences (Weidle et al., (1988) Gene, 73:427-437). The vector for use in transient expression will also often contain a strong promoter such as the SV40 early promoter (e.g., van Zonnenfeld, A. et al., (1987) Proc. Natl. Acad. Sci. USA., 83:4670-4674) to control transcription of the gene of interest. While transient expression provides a rapid method for assay of gene products, the plasmid DNA is not incorporated into the host cell chromosome. Thus, use of transient expression vectors does not provide stable transfected cell lines. A description of a plasmid suitable for transient expression is provided by Aruffo, A., and Seed, B. (1987) Proc. Natl. Acad. Sci. USA., 84:8573-8577.

TM analogs may alternatively be produced in the insect cell lines described above using the baculovirus system. This system has been described by Luckow, V.A., and Summers, M.D. (1988) Bio/Technology, 6:47-55. Generally, this expression system provides for a level of expression higher than that provided by most mammalian systems. The baculovirus infects the host insect cells, replicates its genome through numerous cycles, and then produces large amounts of polyhedron crystals. The



polyhedron gene can be replaced with a TM peptide gene. The polyhedron promoter will then make large amounts of analog protein following infection of the culture host cell and replication of the baculovirus genome. The non-  
5 secreted gene product is harvested from the host 3-7 days post infection. Alternatively, the TM peptide may be secreted from the cells if appropriate signal sequences are present on the protein. The host cells are competent or rendered competent for transfection by  
10 various means. There are several well-known methods of introducing DNA into animal cells. These include: calcium phosphate precipitation, DEAE-dextran technique, fusion of the recipient cells with bacterial protoplasts containing the DNA, treatment of the recipient cells with  
15 liposomes containing the DNA, electroporation and microinjection of the DNA directly into the cells. See, Perbal, B. "Practical Guide to Molecular Cloning," 2nd edition, John Wiley & Sons, New York and Wigler, et al., (1987) Cell, 16:777-785, which are each incorporated  
20 herein by reference.

#### E. Culturing Cells

It is preferred that the host cell is capable of rapid cell culture and able to appropriately glycosylate expressed gene products. Cells, both normal and  
25 transformed, known to be suitable for dense growth in tissue culture are particularly desirable and a variety of invertebrate or vertebrate cells have been employed in the art. In particular, cells which are suitable hosts for recombinant TM expression and which produce or  
30 contain, under culturing conditions, a protease which results in the cleavage of native thrombomodulin now pose no problem in cleaving the mutated protease insensitive TM analog. Examples of such cells include CHO, CHL-1 (characterized as a human melanoma cell), Sf9 cells,  
35 etc., which are publicly available from the ATCC.

The transfected cells are grown up by means well known in the art. For examples, see Kuchler et al. (1977) Biochemical Methods in Cell Culture and Virology. The expression products are harvested from the cell medium in those systems where the protein is secreted from the host cell or from the cell suspension after disruption of the host cell system by, e.g., mechanical or enzymatic means, which are well known in the art.

#### F. Purification of TM Analogs

It is preferred that the TM peptides of this invention be secreted by cultured recombinant eukaryotic cells. The TM analogs are produced in serum-free or serum supplemented media and are secreted intact. If prokaryotic cells are used, the TM analogs may be deposited intracellularly. The peptides may be fully or partially glycosylated or non-glycosylated. Following the growth of the recombinant cells and concomitant secretion of TM analogs into the culture media, this "conditioned media" is harvested. The conditioned media is then clarified by centrifugation or filtration to remove cells and cell debris. The proteins contained in the clarified media are concentrated by adsorption to any suitable resin such as, for example, Q Sepharose or metal chelators, or by use of ammonium sulfate fractionation, polyethylene glycol precipitation, or by ultrafiltration. Other means known in the art may be equally suitable. Further purification of the TM analogs can be accomplished in the manner described in Galvin, J. B., et al., (1987) J. Biol. Chem., 262:2199-2205 and Salem, H.H. et al., (1984) J. Biol. Chem., 259:12246-12251 and in the manner described in the embodiment disclosed herein. The purification of TM analogs secreted by cultured cells may require the additional use of, for example, affinity chromatography, ion exchange chromatography, sizing chromatography, or other conventional protein purification techniques. See, e.g., Deutscher (ed.), "Guide to

Protein Purification" in Methods in Enzymology, Vol. 182 (1990).

Recombinant TM analogs may be found in different forms which are distinguishable under nonreducing chromatographic conditions. Removal of those species having a low specific activity is desirable and is achieved by a variety of chromatographic techniques including anion exchange or size exclusion chromatography. Recombinant TM analogs may be concentrated by pressure dialysis and buffer exchanged directly into volatile buffers (e.g., N-ethylmorpholine (NEM), ammonium bicarbonate, ammonium acetate, and pyridine acetate). In addition, samples can be directly freeze-dried from such volatile buffers resulting in a stable protein powder devoid of salt and detergents. In addition, freeze-dried samples of recombinant analogs can be efficiently resolubilized before use in buffers compatible with infusion (e.g., phosphate buffered saline). Other suitable salts or buffers might include hydrochloride, hydrobromide, sulfate acetate, benzoate, malate, citrate, glycine, glutamate, and aspartate.

#### G. Oxidation Resistant TM analogs

Native thrombomodulin is susceptible to oxidation and when oxidized loses its ability to promote the activation of protein C. Many of the disease conditions requiring anticoagulation are also associated with high levels of toxic oxygen radicals, which can inactivate biomolecules and cause significant tissue damage. Examples of these conditions are reperfusion injury associated with myocardial infarction, DIC associated with septicemia, and alveolar fibrosis associated with adult respiratory distress syndrome. (See, Otani, H., et al., (1984) Circ. Res. 55:168-175, Saldeen, T., (1983) Surg. Clin. N.A. 63(2):285-304, and Idell, S., et al., (1989) J. Clin. Inv. 84:695-705.) In addition, any wound, such as occurring in surgical procedures, involves

the influx of activated monocytes, polymorphonuclear leukocytes, etc., which can create toxic oxygen species as well as releasing a host of proteolytic enzymes, such as elastase. The connection between endothelial cell damage, inflammation, and thrombosis has long been recognized (see The Molecular and Cellular Biology of Wound Repair, ed. Clark, R.A.F. and P.M. Henson (1988), for example). Thrombomodulin is subject to inactivation by exposure to toxic oxygen species and this is expected to have a significant role in many pathogenic states.

Methods for rendering amino acids, specifically methionines, resistant to oxidation are well known in the art. It is possible to chemically modify thiol ether groups with iodoacetic acid, for example, to form oxidation resistant sulphonium groups (Gundlach, H.G., et al., (1959) J. Biol. Chem. 234:1754). A preferred method is by removing the susceptible amino acid or replacing it with one or more different amino acids that will not react with oxidants. The amino acids leucine, alanine, and glutamine would be particularly preferred amino acids because of their size and neutral character. Four methionines of soluble thrombomodulin may be subject to oxidation, particularly those located at residues 291 and 388. If only one methionine is to be blocked or eliminated, it is preferred that it be the residue at position 388.

Methods by which amino acids can be removed or replaced in the sequence of a protein are well known. See, e.g., Sambrook et al., supra; Ausubel et al., supra; U.S. 4,737,462; U.S. 4,588,585; EP-0285 123; and references cited therein. Genes that encode a peptide with an altered amino acid sequence can be made synthetically, for example. A preferred method is the use of site-directed in vitro mutagenesis. Site-directed mutagenesis involves the use of a synthetic oligodeoxy-ribonucleotide containing a desired nucleotide substitution, insertion, or deletion designed to specifically

alter the nucleotide sequence of a single-strand target DNA. Hybridization of this oligonucleotide, also called a primer, to the single-strand template and subsequent primer extension produces a heteroduplex DNA which, when replicated in a transformed cell, will encode a protein sequence with the desired mutation.

To determine the resistance to loss of thrombomodulin activity due to oxidation, the test material (100 - 250  $\mu$ g/ml) is first incubated with an oxidant such as, for example, chloramine-T, hydrogen peroxide at 5-10mM chloramine-T, or 200-1000 mM hydrogen peroxide in a buffer of 0.2% N-ethylmorpholine and 0.008% Tween 80 at pH 7.0, for 20 minutes at room temperature. A buffer of PBS with 0.1% BSA may also be used. After such oxidant exposure, the test material is evaluated using one of the bioactivity assays, e.g., that described below specifically for the ability to act as a cofactor for the activation of protein C. Those mutant TM analogs that retain at least 60%, ordinarily 70%, more normally 80%, and preferably 90%, of activity they had prior to exposure to oxidants are considered to be oxidation resistant as compared to a wild-type (non-mutant) TM analog or native thrombomodulin.

#### H. Laboratory Assays for Measuring TM Activity

A number of laboratory assays for measuring TM activity are available. Protein C cofactor activity can be measured in the assay described by Salem, et al., (1984) J. Biol. Chem. 259(19):12246-12251 and Galvin, et al., (1987) J. Biol. Chem. 262(5):2199-2205. In brief, this assay consists of two steps. The first is the incubation of the test TM analog with thrombin and protein C under defined conditions (see Examples below). In the second step, the thrombin is inactivated with hirudin or antithrombin III and heparin, and the activity of the newly activated protein C is determined by the use of a chromogenic substrate, whereby the chromophore is

released by the proteolytic activity of activated protein C. This assay is carried out with purified reagents.

Alternatively the effect of a TM analog can be measured using plasma in clotting time assays such as the activated partial thromboplastin time (APTT), thrombin clotting time (TCT) and/or prothrombin time (PT). The APTT assay is dependent on both the activating of protein C, as well as the direct inhibition of thrombin, while the TCT and PT assays are dependent only on the inhibition of thrombin. Prolongation of the clotting time in any one of these assays demonstrates that the molecule can inhibit coagulation in plasma.

The above assays are used to identify soluble TM analogs that are able to bind thrombin and to activate protein C in both purified systems and in a plasma milieu. Further assays are then used to evaluate other activities of native thrombomodulin such as inhibition of thrombin catalyzed formation of fibrin from fibrinogen (Jakubowski, et al., (1986) J. Biol. Chem. 261(8):3876-3882), inhibition of thrombin activation of Factor V (Esmon, et al., (1982) J. Biol. Chem. 257:7944-7947), accelerated inhibition of thrombin by antithrombin III and heparin cofactor II (Esmon, et al., (1983) J. Biol. Chem. 258:12238-12242), inhibition of thrombin activation of Factor XIII (Polgar, et al., (1987) Thromb. Haemostas. 58:140), inhibition of thrombin mediated inactivation of protein S (Thompson and Salem, (1986) J. Clin. Inv. 78(1):13-17) and inhibition of thrombin mediated platelet activation and aggregation (Esmon, et al., (1983) J. Biol. Chem. 258:12238-12242).

In the present invention, the TM analogs do not necessarily have all activities equal to that of native thrombomodulin. For example, if one compares an amount of a TM analog of the present invention with an equivalent amount of native chondroitin sulfonated membrane bound thrombomodulin (as measured in units of protein C cofactor activity, defined below) the TM analog

will usually have at least a 20% reduction, and preferably a 50% reduction in its ability to inhibit thrombin-mediated conversion of fibrinogen to fibrin compared to the native thrombomodulin.

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I. Methods for Altering the Glycosylation of TM Analogs

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Carbohydrate substituents on proteins can affect both biological activity and circulating half-life. In order to make some TM analogs of the present invention, O-linked glycosaminoglycan carbohydrate such as is found in the native thrombomodulin protein, should be eliminated. There are numerous ways of accomplishing this objective. One method would be the treatment of the O-linked carbohydrate containing protein with a glycohydrolase known to specifically degrade sulfated glycosaminoglycans, such as chondroitinase ABC or hyaluronidase. This method is described in Bourin, M. et al., (1988) J. Biol. Chem. 263(17):8044-8052, which is herein incorporated by reference.

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35

A second method for eliminating the O-linked carbohydrate is by introducing site directed mutations into the protein. The attachment of glycosaminoglycans is typically directed by a consensus recognition sequence of amino acids X-serine-glycine-X-glycine-X (Bourdon, M.A., et al., (1987) Proc. Natl. Acad. Sci. USA. 84:3194-3198) where X is any amino acid. The recognition sequence for other types of O-linked sugars is generally threonine/serine-X-X-proline. The O-linked domain of natural thrombomodulin normally has at least one potential glycosaminoglycan addition site (aa 472 and/or 474) and four other potential O-linked carbohydrate addition sites (aa 464, 472, 474, 480 and 486), depending upon the cell type. Any change introduced into the nucleotide sequence that removes or changes the identity of any one or more of the amino acids in this recognition sequence might eliminate the potential O-linked carbohydrate attachment site. Methods of introducing site directed mutations

into a nucleotide sequence are described above. For example, one or more of these serine or threonine residues may be modified to an unglycosylatable amino acid, e.g., alanine.

5 A preferred method of eliminating O-linked carbohydrate from a TM analog is by making an analog peptide that does not include the amino acids that are considered to be the O-linked domain, i.e., amino acids 468 through 485 of the native thrombomodulin gene sequence as shown  
10 in Table 1. Methods of accomplishing this are well known in the art and have been described above.

The circulating half-life of a protein can be altered by the amount and composition of carbohydrate attached to it. The TM analogs of the present invention  
15 contain both O-linked and N-linked carbohydrate. In addition to the potential O-glycosylation sites discussed above there are potential N-linked sites, e.g., at amino acids 29, 97, 98, 364, 391 and 393, and potential O-linked sites, e.g., at amino acids 319, 393 and 396.  
20 Methods of altering carbohydrate composition in addition to those described above are: 1) expression of the TM analog gene in bacteria such *E. coli*, which does not have the cellular mechanisms necessary to glycosylate mammalian proteins, 2) expression of the TM analog gene in  
25 various eukaryotic cells, as each has its own characteristic enzymes that are responsible for the addition of characteristic sugar residues, and 3) treatment with chemicals such as hydrofluoric acid. Hydrofluoric acid, for example, chemically digests acidic and neutral sugars  
30 while leaving intact sugars such as N-acetyl glucosamines and, under certain conditions, galactosamines.

#### J. Protease resistant analogs

As noted above, when recombinantly produced TM is expressed in culture, especially in eukaryotic cells,  
35 e.g., CHO cells, the TMs contain substantial amounts of a two-chain version. This is detectable, e.g., by a



binding property profile which indicates that there is a species with a different binding constant present, due to cleavage of the heterologous protein by an endopeptidase present under culturing conditions or during the purification process. This endopeptidase may be a constituent of, e.g., the host cell, a microbiological contaminant, the culture medium, etc. Analysis on SDS-PAGE under reducing conditions reveals that, when TM analogs are prepared in CHO cells and isolated from the conditioned media as disclosed herein, a protein band corresponding to a molecular weight of about 80 kD is present in addition to the 94 kD protein band which corresponds to single-chain soluble TM analog, as determined using rabbit polyclonal antibodies to insect cell 6EGF, e.g., TM<sub>2</sub>(Sf9), or stained gels. However, the same material expressed in CHL-1 cells apparently has less of this degraded material. This indicates that the extent of proteolytic activity may be cell-line dependent.

A unique protease cleavage site in the human thrombomodulin extracellular domain has now been identified in various human analogs. In addition to the expected heterogeneity in the N-terminus (both FPAPAEP and APAEP N-termini are found) caused by signal sequence cleavage heterogeneity. These analogs contain a new sequence IGTD\_D\_K, which is consistent with proteolysis of single-chain TM between Arg<sup>456</sup> and His<sup>457</sup>. The amount of protein present in this cleaved form is as high as 50% of the TM chains.

This proteolytic cleavage site is formed in the last (c) loop of the sixth EGF domain, and thus the protein fragment is covalently held together by the last disulfide bond in the loop. This is further supported by the fact that the two bands are only seen on reducing gels. The 80 kDa band is the N-terminal fragment, based upon size and immunoreactivity. Therefore, TM analogs expressed in CHO cells, as well as in other cell lines which possess similar proteases that degrade the TM

analog, are contaminated with cleaved two-chain material that exhibits similar molecular properties, e.g., molecular weight and amino acid sequence when assayed under the same conditions. Therefore, purification properties will often be similar, even though the TM analog is cleaved into a two-chain version, which is held together by disulfide bond(s).

As was noted, this cleavage site occurs in the c loop of the 6EGF domain of TM. Deletion of this loop in various constructs was shown to result in substantial loss in thrombin binding, evidenced by a large (~ 7-fold) increase in  $K_d$  for thrombomodulin. Therefore, the contaminating two-chain material likely results in a similar loss of specific activity of such preparations. Furthermore, since other binding sites on the molecule are intact, the two-chain material should act as a competitive inhibitor of the single-chain TM analog. It is very difficult to separate two-chain material from the intact one-chain species, so it is important to produce highly homogeneous, intact single-chain TM which is not subject to proteolytic cleavage. This result has been accomplished through the construction of TM analogs, as disclosed herein, which remove and/or replace proteolytic cleavage sites and thus solve this heretofore unappreciated problem. The present invention allows for providing both full-length membrane-associated or soluble TM analogs which are resistant to cleavage by said protease(s).

Mutations can be routinely introduced into the TM analogs to modify the protease cleavage site in accordance with the procedures described herein. However, the c loop of 6EGF has been shown to be important in thrombin binding, and it is important that the binding properties of this area be maintained in the analog produced. The biological activity of the thus-obtained molecule would be maintained, or an increase in overall activity of a TM analog composition achieved, by preventing proteolysis. This will avoid loss of activity caused by change in the

structure of the initial domain thought to be important in binding TM to thrombin. For example, since in rabbits, mice, and cows the 456-457 sequence of TM is Gly-Gln instead of Arg-His, as in the human TM, this modification is of particular interest. Other, similar, site specific mutations can be employed to routinely identify a modified sequence which is not subject to proteolysis, yet maintains the desired level of biological activity. For example, by homology to other similar EGF-like proteins, it can be seen that this region of the molecule is most likely to be in a  $\beta$ -sheet structure between reverse turns around Pro<sup>450</sup>/Asp<sup>451</sup> and Thr<sup>460</sup>/Asp<sup>461</sup>. By this analysis, especially favorable substitutions would be those which incorporate amino acids found in high frequency in  $\beta$ -sheet structures, e.g., His, Val, Ile, Phe, Tyr, Trp, and Thr. Other favorable substitutions would be those which incorporate amino acids less likely to be found in  $\beta$ -sheet structures, e.g., Cys, Glu, Lys, Asp, Asn, and Pro. Certain other residues include, e.g., Cys, to prevent incorrect disulfide bonding with the other, structurally important, cysteines; Pro, which is less consistent with a  $\beta$ -sheet structure; and Lys and Arg, which may incorporate alternative, protease cleavage sites. Thus, one of ordinary skill in the art can easily and routinely determine the structure of TM analogs which will be resistant to protease cleavage, at this site or any other protease cleavage site. All such structures meeting the requirements described herein are included as part of this invention.

Of course, this modification can be employed in addition to one or more of the other modifications disclosed herein, e.g., for introducing oxidation resistance, increasing half-life of the analog in serum by removing glycosylation sites, homogeneous amino termini, homogeneous carboxy termini, etc., to provide a molecule having improved characteristics at several sites.

In addition to the previously described modifications, single-chain TM can be provided by removal of two-

chain TM from preparations which contain it by routine protein purification methods.

K. Production of thrombomodulin analogs  
having a unique N-terminus

5 In addition to the above problem of preventing the production of TM polypeptide compositions exhibiting more than one N-terminus due to internal endoproteolytic cleavage, native thrombomodulin has an additional inherent heterogeneity at the normal N-terminus due to imprecise processing of the signal sequence. One characteristic of  
10 purity used in the definition of protein purity, e.g., in particular for regulatory approval for in vivo administration, is the detection of a single unique N-terminal sequence. It is commercially and otherwise advantageous to be able to produce a TM polypeptide composition having  
15 a unique N-terminal processing site, so as to avoid any potential ambiguity regarding the nature of the final product. Therefore, the nucleotide sequence encoding the N-terminal region of the polypeptide can be modified such that the processing enzyme(s) of the host cell will generate a single N-terminus of the mature polypeptide. This  
20 can be accomplished, e.g., by deleting the N-terminal three amino acids which constitute one of the processing sites, which results in a fully functional polypeptide that starts at amino acid 4 (Glu) of the native TM. This  
25 further provides a polypeptide having only a single and unique post-processing N-terminus. Other functional constructs can also be prepared routinely, having other single N-termini, either through further deletion of the  
30 native TM N-terminus or through substitution of alternative homogeneous N-terminal processing sites.

L. Production of thrombomodulin analogs  
having a unique C-terminus

35 A still further modification which can be made in accordance with the goals of this invention is to modify the TM analog composition so it exhibits a unique carboxy

terminus. Providing single-chain material ensures elimination of at least one C-terminus in accordance with the procedures disclosed herein. One particularly advantageous construct provides a TM analog which is resistant to proteases or other factors, e.g., post-translational processing enzymes or C-terminal exoproteases, by providing a C-terminus which is resistant to degradation. These analogs are provided by modifying the DNA coding for the C-terminal amino acids of the polypeptide. For example, as disclosed herein, the C-terminus of the functional analog ending at amino acid 497 of native TM can conveniently be shortened by 7 amino acids to provide a C-terminus ending in a -Pro-Pro sequence which is particularly protease-resistant. Other amino acid deletions and substitutions which provide biologically active TM analogs can be prepared in accordance with routine modifications of the methods known in the art and as described herein.

M. Formulation and Use of Thrombomodulin Analogs

The soluble TM analogs described herein may be prepared in a lyophilized or liquid formulation. The material is to be provided in a concentration suitable for pharmaceutical use as either an injectable or intravenous preparation.

These compounds can be administered alone or as mixtures with other physiologically acceptable active materials, such as antibiotics, other anticoagulants, one-chain t-PA, or inactive materials, or with suitable carriers such as, for example, water or normal saline. The analogs can be administered parenterally, for example, by injection. Injection can be subcutaneous, intravenous or intramuscular. These compounds are administered in pharmaceutically effective amounts and often as pharmaceutically acceptable salts, such as acid addition salts. Such salts can include, e.g., hydrochloride, hydrobromide, phosphate, sulphate, acetate,

benzoate, malate, citrate, glycine, glutamate, and aspartate, among others. See, e.g., Remington's Pharmaceutical Sciences (17th ed.), Mack Publishing Company, Easton, Pennsylvania, and Goodman & Gilman's, The Pharmacological Basis of Therapeutics, 8th ed., Pergamon Press, 1985, both of which are herein incorporated by reference. The analogs described herein may display enhanced in vivo activity by incorporation into micelles and/or phospholipid aggregates. Methods for incorporation into ionic detergent aggregates or phospholipid micelles are known.

An antithrombotic agent can be prepared using the soluble TM analogs described herein and can consist of a completely purified analog alone or in combination with a thrombolytic agent as described above. Compounds of the present invention which are shown to have the above recited physiological effects can find use in numerous therapeutic applications such as, for example, the inhibition of blood clot formation. Thus, these compounds can find use as therapeutic agents in the treatment of various circulatory disorders, such as, for example, coronary or pulmonary embolism, strokes, as well as the prevention of reocclusion following thrombolytic therapy, and these compounds have utility in the cessation of further enlargement of a clot during an infarction incident. Further, the compounds disclosed can be useful for treatment of systemic coagulation disorders such as disseminated intravascular coagulation (DIC), which is often associated with septicemia, certain cancers and toxemia of pregnancy.

These compounds can be administered to mammals for veterinary use, such as with domestic animals, and for clinical use in humans in a manner similar to other therapeutic agents, that is, in a physiologically acceptable carrier. In general, the administration dosage for the TM analog will range from about at least 0.0002, more usually 0.02, and less than 5000, usually less than 2000,

more usually less than 500  $\mu\text{g/kg}$ , usually 0.02 to 2000  $\mu\text{g/kg}$  and more usually 0.02 to 500  $\mu\text{g/kg}$  of the host body weight. These dosages can be administered by constant infusion over an extended period of time, until a desired circulating level has been attained, or preferably as a bolus injection. Optimal dosages for a particular patient can routinely be determined by one of ordinary skill in the art.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative and not limitative of the remainder of the disclosure in any way whatsoever.

In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

The entire disclosures of all applications, patents, and publications, cited above and below, are hereby incorporated by reference.

## EXAMPLES

The first four examples which follow are directed primarily toward TM<sub>E</sub>(Sf9). Similar methods are applicable with routine modifications to the TM<sub>LEO</sub> made in mammalian cells, as described in the later examples.

**Example 1: Isolation and expression of TM analog sequences**

**A. Cloning**

Genes for producing recombinant thrombomodulin analog peptides were isolated as described in copending applications U.S. Serial No. 345,372, filed 28 April 1989, U.S. Serial No. 406,941, filed 13 September 1989, and PCT Serial No. 90/00955, filed 16 February 1990, each herein incorporated by reference. Briefly, human DNA was used to isolate a gene encoding the 6 EGF-like domains of thrombomodulin corresponding to amino acids 227-462 as well as other portions of the thrombomodulin peptide. (See Table 1). This DNA was isolated from fetal liver according to the method of Blin, N and DW Stafford, (1976) *Nucleic Acids Res.* 3:2302. The DNA was then used as a template in a polymerase chain reaction with synthetically derived primers selected to embrace the desired regions (See Tables 3 & 4). See, e.g., Innis et al., *PCR Protocol, A Guide to Methods and Applications* (1990), Academic Press.

**1. Isolation of genes encoding amino acids 227-462**

The following steps provide a means to obtain a DNA insert encoding amino acids (aa) 227-462 and uses primers #1033 and #1034 (See Table 3). It is understood that by modifying the procedures set forth below by using alternative primers, other soluble TM analogs can be obtained.

The sequence of the #1033 and #1034 primers correspond to the 5' and 3' ends of the desired domain, but they have been modified so that they contain a BamHI



site. A termination codon (TGA) was introduced following base 1586. The polymerase chain reaction was run under the conditions described by Saiki, et al., (1988) Science 320:1350-1354, except that the initial temperature of annealing was 37° C. After 10 cycles, the annealing temperature was raised to 45° C for the remaining 30 cycles. An aliquot of the reaction products was separated on a 5% polyacrylamide gel and visualized by ethidium bromide staining. A band of the predicted size (700 bp) could clearly be seen. Alternatively one can sequence this band or hybridize it to an internal probe to confirm its identity.

2. Isolation of genes encoding other regions of thrombomodulin

The polymerase chain reaction as herein described was used in the same manner to isolate additional fragments of thrombomodulin corresponding to the regions listed in Table 4. In particular, these regions embrace one or more of the EGF-like domains and the O-linked glycosylation domain. The sequences of the primers selected to produce the desired regions are shown in Table 3.

3. Cloning plasmids containing the thrombomodulin analog genes

The remainder of the polymerase chain reaction mixture described above (i) was restricted with BamHI, separated on a 5% polyacrylamide gel, and the 700 bp band was excised and eluted. It was ligated to pUC19 that had been restricted with BamHI and the new plasmid was transformed into *E. coli* strain DH5-alpha. Recombinant colonies were selected on a medium containing ampicillin and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside. White colonies were picked onto a grid and hybridized by the Grunstein-Hogness technique with a synthetically derived gene corresponding to aa 283-352 of thrombomodulin that had been cut out of a cloning plasmid (pTM2.1) with EcoRI

and HindIII before labelling with  $^{32}\text{P}$  by random priming (Boehringer Mannheim).

After exposing the filters to X-ray film the one colony that hybridized to the pTM2.1 probe was selected and a culture grown up. DNA was extracted and analyzed by restriction with either BamHI or BglII to confirm the presence of an insert with the correct restriction map. The excised insert was also transferred to nitrocellulose and analyzed by hybridization with labelled pTM2.1. Both methods confirmed that the 700 bp insert contained the coding sequence for the 6 EGF-like domains of thrombomodulin. The insert was sequenced to verify that no mutations had been inadvertently introduced during the PCR procedure. The plasmid containing the desired gene fragment is named pUC19pcrTM7.

#### B. Expression of TM

##### 1. Construction of AcNPV Transfer Vectors

The transfer vectors described below are also described in copending application U.S. Serial No. 07/812,892, which is a continuation of U.S. Serial No. 07/345,372. The transfer vectors contain the Hypodermin A signal sequence from *Hypoderma lineatum*.

##### i. pHY1 and pSC716

Oligomers containing the Hypodermin A signal sequence, a translation initiation codon, a BglII cloning site, a BamHI 5' overhang and a KpnI 3' overhang, COD#1198 and COD#1199 (see Table 2), were annealed and cloned into pSC654, a pUC19 derivative, creating pHY1. Plasmid pHY1 was restricted with BamHI and EcoRI, releasing the hypodermin A signal sequence. This sequence was then ligated to pSC714 to create the vector pSC716. Plasmid pSC714 is a derivative of pVL1393, obtained from Summers, et al. The only difference between the two is that in pSC714, one of the BglII sites has been destroyed.

ii. Construction of pPHY101

The BamHI fragment from pUC19pcrTM7 (see Aiii above) was cloned into the BglII site of pPHY1 and the orientation was chosen such that the hypodermin A signal sequence was adjacent to amino acid 227. This plasmid is pPHY101.

iii. Construction of the AcNPV transfer vector pTMHY101

Plasmid pPHY101 was treated with BamHI/EcoRI which releases the Hypodermin A signal sequence linked to the TM analog coding sequence. Shuttle vector pVL1393 contains a partially deleted AcNPV polyhedrin gene and unique BamHI and EcoRI cloning sites. The BamHI/EcoRI fragment from pPHY101 was inserted downstream of the polyhedrin promoter, thus creating a plasmid, pTMHY101, in which the hybrid gene was under the control of the polyhedrin promoter.

iv. Construction of other ACNPV transfer vectors

Transfer plasmids containing other TM analog gene sequences were constructed using a strategy similar to that outlined above. Fragments from the cloning plasmids described above were cloned into pSC716 in frame so that the TM analog gene sequence was fused to the hypodermin A signal sequence. The TM gene sequences are listed in Table 4.

v. Production of pure phage stocks

Cell transfection was done using a calcium phosphate precipitation technique modified for insect cells according to Summers and Smith. Briefly, a T25 flask was seeded with  $2 \times 10^6$  Sf9 cells, and the cells were allowed to attach for one hour at room temperature. Two  $\mu$ gs of transfer vector, for example, pTHR28, and 1  $\mu$ g of AcNPV DNA were coprecipitated in calcium phosphate and incubated with the cells for 4 hours. The cells were

rinsed and re-fed with growth media, then placed in a 28° C incubator for 3-4 days. During this incubation, the cells produce both recombinant and non-recombinant virus which accumulate in the growth media. This media, containing a mixed viral stock, was assayed for the presence of protein C cofactor activity (see below).

Recombinant viruses were detected by plaque assay. The transfection stocks were diluted ( $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$ ) and plated 4-7 days post-transfection. Occlusion negative (recombinant) plaques were picked 7 days after plating and replated ( $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$  dilution). After another 7 days, the plates showed 100% pure occlusion-negative recombinant plaques. A single pfu from each was selected for production. A high titer viral stock was grown by infecting 5 mls of Sf9 cells ( $1 \times 10^6$ /ml in Excell 400 medium (JR Scientific)) with a single pfu, grown for 4-5 days. A portion of this stock was then diluted 1:50 - 1:100 into Sf9 cells grown to mid-log phase to produce a protein stock.

## 2. Production of Human TM Analogs in Mammalian Cells

### i. Mammalian expression vectors for TM analogs

This example provides a mammalian expression vector comprising the analog genes of Example 1, A. Genes can be operably linked to the signal sequence of human tissue plasminogen activator (see Table 2). The expression plasmid, pPA124, contains a promoter contained within the three copies of the long terminal repeats derived from Harvey Sarcoma virus for the expression of cloned genes. This plasmid was derived from pPA119, and pSC672, both described in detail in co-pending U.S. Serial No. 251,159, filed September 29, 1988, incorporated herein by reference. A BglII - BclI fragment containing the SV40 polyadenylation region was isolated from pSC672. This fragment was cloned into pPA119 which had been digested with BglII and BclI. In the resulting plasmid, pPA124,

both the BglII and BclI sites remained intact. Plasmid pPA124 contains the t-PA signal sequence adjacent to an appropriate restriction site and this signal sequence also contains the second intron of the human t-PA gene.

5 The gene encoding the soluble TM analog was removed from pUC19pcrTM7 by treatment with BamHI and ligated to pPA124 that had been treated with BglII. Transformants were screened for the presence of the insert in the correct orientation, that is, in which the t-PA signal  
10 sequence was linked to the 5' end of the thrombomodulin insert encoding an open reading frame. This plasmid, pTM101, was then digested with ClaI and ligated to a ClaI fragment containing the dhfr gene under the control of the SV40 promoter. The ClaI fragment is described in  
15 WO88/02411 at page 26. Transformants were screened for the presence of this dhfr cassette and then the orientation relative to the plasmid was determined by restriction mapping (pTM103).

Plasmid pTM103, containing the dhfr sequence in the  
20 divergent direction to the thrombomodulin sequence, was treated with BclI. A DNA fragment encoding a gene providing hygromycin resistance on a BamHI fragment was ligated into the plasmid. Clones were selected, after transformation into *E. coli* strain DH5 $\alpha$ , by their ability  
25 to grow on plates containing both ampicillin and hygromycin B. The orientation of the hygromycin B gene relative to the plasmid was determined by restriction mapping. One plasmid, pTM108, in which the hygromycin B gene lies in the opposite orientation to the TM gene, was  
30 grown up in culture. This plasmid has the sequences encoding the TM analog under the control of the triple LTR promoter, with both a gene that confers hygromycin B resistance and one that encodes dhfr present on the plasmid. A similar expression plasmid, pTHR13, also  
35 contains the t-PA signal sequence operably linked to the sequence encoding the 6 EGF-like domains both under the control of the cytomegalovirus promoter. This plasmid

contains the M13 origin of replication making it useful for site directed in vitro mutagenesis, described below. The thrombomodulin sequence was linked to the tissue plasminogen activator signal sequence, ensuring its secretion. The TM analog produced by both these plasmids, 4t/227-462, is comprised of the 6 EGF-like domains of thrombomodulin with an additional 4 amino acids on the N-terminal end that are the result of processing of the t-PA signal peptide.

10           ii. Transfection, selection and amplification of stable mammalian clones

For the transfection, 10 µg of pTM108 was mixed with Lipofectin reagent (Bethesda Research Laboratories) and added to a monolayer of 10<sup>5</sup> CHL-1 host cells in 6-well plates. Forty-eight hours after transfection, a known number of cells were plated onto selective media. Resistance to hygromycin B was used as the selection marker. CHL-1 cells transfected with the bacterial hygromycin B gene can survive growth in 0.3 mg/ml hygromycin B.

20           The transfection or selection frequency was 2/10<sup>3</sup> and was determined as the number of colonies arising after selection, divided by the total number of cells plated. The culture supernatant was shown to contain 1.5 U/ml TM activity after 24 hours in contact with the cells.

25           A population of cells resistant to the first selection conditions were then subjected to a second round of selective pressure. Either 100 nM or 500 nM methotrexate (MTX) was added to the growth medium to select for transfectants that expressed the dhfr gene. Only clones which had amplified the dhfr gene would be able to grow in this high level of MTX. In the process of gene amplification, other plasmid sequences will be co-amplified with the dhfr gene and thus lead to increased gene expression of the non-selectable gene as well. Resistant clones were apparent after 5 to 6 weeks. Individual clones resistant to these levels of MTX were isolated and assayed. A culture after selection in 100

nM MTX was shown to produce 4.9-14.7 U per ml of protein C activating activity (see below). A pooled population was plated into a ten-fold greater concentration of MTX (1  $\mu$ M or 5  $\mu$ M). Clones were again recovered from this selection step and assayed. At each step clones were shown to produce and secrete TM analog into the culture medium.

### C. Site-directed Mutagenesis

The 6 EGF-like domains region of native thrombomodulin has two methionine residues, one at position 291 and one at position 388. (See Table 1). Site-directed in vitro mutagenesis was used to convert either or both of these methionines to other amino acids. Site-directed mutagenesis uses a synthetic DNA sequence containing a desired nucleotide substitution, insertion, or deletion to specifically alter the nucleotide sequence of a single-stranded template DNA. Hybridization of this synthetic DNA to the template and subsequent primer extension produces a heteroduplex DNA capable of cell transformation to yield the desired mutation. A diagram depicting this process is shown in Figure 1 of U.S. Patent Application Serial No. 07/568,456.

A similar method can be used to eliminate potential protease-sensitive regions, to modify glycosylation sites, and to modify the amino acid carboxy termini of the desired TM analog.

A plasmid for making single stranded DNA copies, pTHR14, was constructed by ligating the F1 origin of replication contained on an AseI-ScaI fragment into an insect cell transfer vector, pTMHY101, previously digested with NdeI and ScaI. Plasmid pTMHY101 contains a gene sequence that produces a peptide corresponding to the 6 EGF-like domains of thrombomodulin, amino acids 227-462, and is described above. pTMHY101 is described in copending application U.S. Serial No. 345,372 as well as B(1)(iii) above. A similar vector containing the

lectin domains, 6 EGF, and O-linked domains was used for mutagenizing and constructing the pTHR525, which encodes one preferred embodiment.

Specific mutagenizing oligonucleotide primers were synthesized and used with the MUTATOR™ - DNA Polymerase III Site-directed Mutagenesis Kit (Catalogue #200500, Stratagene, La Jolla, CA), except as otherwise noted, to prime second strand synthesis and create thrombomodulin analog genes with either one or both of the methionines changed to a non-oxidizable amino acid. Primers directing conversion to the preferred amino acids leucine, glutamine or alanine are shown in Table 5. Also included in these primers are substitutions in the nucleotide sequence that add a unique restriction enzyme site useful as a diagnostic for successful mutagenesis but which do not necessarily change the corresponding amino acid sequence. The nucleotide substitutions are underlined in the primers shown in Table 5. For example, in plasmid pTHR28 the methionine at position 388 in the native thrombomodulin protein was replaced with leucine, and in the process a unique PvuII site was introduced. It is understood that other substitute non-oxidizable amino acids would be equally useful in this embodiment of the present invention.

Purified single-stranded DNA templates were prepared using the procedure described by Bio-Rad (Muta-Gene Phagemid *in vitro* Mutagenesis, Instruction Manual, Cat. no. 170-3576, pages 33-34) although other procedures known in the art would be equally suitable.

The 5' terminus of each mutagenizing primer was phosphorylated by incubating 0.5 ng/ $\mu$ l of primer in a solution containing 2 mM rATP, 0.4 U/ $\mu$ l polynucleotide kinase in annealing buffer (20 mM Tris-HCl pH 7.5, 8 mM MgCl<sub>2</sub>, and 40 mM NaCl) at 37° C for 30 minutes. The reaction was heat inactivated by incubating the mixture at 65° C for 15 minutes. Phosphorylation increases the rate of successful mutation. The phosphorylated primer



was annealed to the single-stranded template by heating 100 ng of template and 2.5 ng of primer in 25  $\mu$ l of annealing buffer to 65° C for 5 minutes then allowing the mixture to cool and anneal at room temperature for 10 minutes. Double stranded DNA was made by primer extension essentially as described by Tsurushit, N., et al., (1988) Gene 62:135-139, and O'Donnell, M.E., et al., (1985) J. Biol. Chem. 260:12875-12883. Briefly, the template/primer mixture was diluted (1:1) with 10% annealing buffer plus 80  $\mu$ g/ml bovine serum albumin, 2.5 mM dithiothreitol, 0.25 mM mixed dNTPs, 2 mM rATP and 1% glycerol plus 1  $\mu$ g of single-stranded DNA binding protein. The reaction was incubated for 5 minutes at room temperature to allow the binding protein to coat the single-strand DNA template. DNA polymerase III holoenzyme (*E. coli*, 1.7  $\mu$ l of 50 U solution) was added, and the reaction was incubated at 30° C for 10 minutes. T4 DNA ligase was added (0.5  $\mu$ l, 2 Weiss units) and the reaction was further incubated for 5 minutes at 30° C. This mixture was used to transform *E. coli* and properly mutated clones were selected by restriction digest pattern.

This same process can be used to make mutants that can be expressed in mammalian cells using, for example, pTR13 (described above) which has an M13 origin of replication for making single stranded DNA templates.

#### **D. Production and purification of recombinant protein**

T25 flasks were seeded at a density of  $2 \times 10^6$  Sf9 cells in 5 ml TMN-FH media plus 10% FBS or Excell 400, then infected with an isolated recombinant plaque from Part B or C above. Viral stocks were collected after three days. Flasks (30-100 ml shaker flasks or 100-300 ml spinner flasks) were seeded with cells ( $1-1.8 \times 10^6$ /ml) and infected with aliquots of the viral stock equal to 1/50th to 1/100th of the final volume. The infected cell cultures were grown for four days before harvesting the

conditioned media containing recombinant oxidation resistant TM analog protein.

The TM analogs were purified from conditioned media by removal of cell debris, followed by five chromatography steps: 1) Q Sepharose, 2) thrombin affinity, 3) gel filtration, 4) anion exchange, and 5) a second gel filtration step. The gel filtration steps effect an exchange of buffers. All chromatography steps were performed at 4° C.

#### 1. Materials

Some of the chromatographic resins were purchased from commercial sources. Q Sepharose and Sephadex G25 were purchased from Sigma (St. Louis, MO), and Mono Q 5/5<sup>®</sup> from Pharmacia LKB (Piscataway, NJ).

DFP-thrombin agarose was prepared approximately as follows: 360 mg of bovine thrombin in 100 ml of 20 mM Na phosphate, pH 7.5 was added to approximately 100 ml of a 50% Affigel 10 resin slurry and mixed overnight at 4°C. The Affigel 10 was prepared for use as described by the manufacturer and equilibrated with the load buffer. Residual active esters were blocked by the addition of 100 ml of 0.1 M glycine methylester (pH 5.6) for one hour at 4°C. The gel was then equilibrated with 30 mM Tris-HCl, 2 M NaCl, pH 7.5, and 20 µl of DFP was added to give a final concentration of about 1 mM DFP. After 16 hrs of mixing at 4°C an additional 6 µl of DFP was added and mixing continued for 4 additional hours. The resin was then washed with 20 mM Tris-HCl, 2 M NaCl pH 7.5 and stored at 4°C.

Thrombin activity was measured using the Kabi S-2238 substrate and indicated that >86% of the thrombin was removed from the solution, and presumably coupled to the resin, giving a final concentration of about 6 mg of thrombin per ml of resin. The enzymatic activity of the DFP treated resin was <1% of the starting activity.

## 2. Production of pure TM<sub>p</sub> analog peptide

Conditioned media was harvested and clarified by centrifugation at 1400 x g for 10 minutes. The pH was adjusted from about 6.0 to about 5.2 with glacial acetic acid. The adjusted media was then loaded onto a column of Q Sepharose resin. The column had previously been equilibrated with about four column volumes of wash buffer 1 (117 mM Na acetate, 0.02% NaN<sub>3</sub>, pH 5.0). After loading, the column was washed with wash buffer 1 followed by wash buffer 2 (25 mM Na acetate, 0.1 M NaCl pH 5.0) then the oxidation resistant TM analog was eluted with wash buffer 2 containing 0.3 M NaCl, pH 5.0.

Column fractions containing activity as measured in the protein C activation assay (see above) were pooled, then diluted with of 0.3 M NaCl, 20 mM Tris-HCl, 0.5 mM CaCl<sub>2</sub>, 0.02% NaN<sub>3</sub>, pH 7.5. The pH of the diluent was measured and adjusted to about 7.5 with NaOH. The ionic strength of the pool was about the ionic strength of a solution of 0.3 M NaCl. This adjusted pool was loaded overnight by gravity onto a thrombin agarose column pre-equilibrated with the same buffer used to dilute the conditioned media. The column was washed with diluent buffer, and the TM analog was removed from the matrix with 2.0 M NaCl, 20 mM Tris HCl, 1 mM NaEDTA, 0.02% NaN<sub>3</sub>, pH 7.5.

The substantially pure TM analog was applied to a Sephadex G25 column and recovered in 0.2% N-ethylmorpholine acetate (NEM) pH 7.0. This step removes NaCl.

TM analog collected from the Sephadex G25 column was applied to a Mono Q column (Pharmacia, 10 micron particles, quaternary amine) pre-equilibrated with 0.2% N-ethylmorpholine (NEM) pH 7.0. After washing with this buffer the various forms were separated using a gradient of 0 to 0.4 M NaCl. Samples of each fraction were evaluated on an SDS-PAGE gel under non-reducing conditions. SDS Polyacrylamide Gel Electrophoresis was performed by the method of Laemmli using 3.3% acrylamide in the

stacking and 12.5% acrylamide in the running gel. Nonreduced samples were diluted in Laemmli sample solubilization buffer (50 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, and 0.01% bromophenol blue) and loaded directly onto the gel. Pharmacia LMW Calibration Kit protein standards were used for MW markers, and the gels were silver stained. In some fractions, only one major band is visible with silver staining.

Fractions containing peptides with like mobilities were pooled and then assayed for total protein content and for activity in the protein C activation assay as described below.

#### E. Assays for Thrombomodulin Analogs

##### 1. Materials

Rabbit thrombomodulin, hirudin and human Protein C were obtained from Integrated Genetics or American Diagnostica. Human thrombin is available from a variety of noncommercial and commercial sources. Bovine thrombin for affinity chromatography was purchased from Miles Labs, Dallas, Texas. D-valyl-L-leucyl-L-arginine-p-nitroanilide (S-2266) and D-Phe-Pip-Arg-p-nitroanilide (S-2238) were purchased from Kabi Vitrum.

Bovine serum albumin (fraction V), citrated human plasma, and APTT reagent were purchased from Sigma Chemicals. Microtiter plates were supplied by Dynatech (#25861-96). All other reagents were of the highest grade available.

##### 2. Methods and Results

##### i. Protein C Activation Assay (Chromogenic)

This assay was performed by mixing 20  $\mu$ l each of the following proteins in a microtiter plate: thrombomodulin sample (unknown or standard), thrombin (3 nM), and Protein C (1.5  $\mu$ M). The assay diluent for each protein was 20 mM Tris-HCl, 0.1 M NaCl, 2.5 mM CaCl<sub>2</sub>, 5 mg/ml BSA, pH 7.4. The wells were incubated for up to 2 hours at

37°C, after which Protein C activation was terminated by the addition of 20  $\mu$ l of hirudin (0.16 unit/ $\mu$ l, 570 nM) in assay diluent and incubation for an additional 10 minutes.

5       The amount of activated Protein C formed was detected by adding 100  $\mu$ l of 1.0 mM S-2266 (in assay diluent), and continuing to incubate the plate at 37°C. The absorbance at 405 nm in each well was read every 10  
10       seconds for 15 minutes, using a Molecular Devices plate reader. The absorbance data was stored, and the change in absorbance per minute (slope) in each well was calculated. The change in absorbance per minute is proportional to pmole/ml of activated Protein C.

15       This ratio was determined empirically using varying concentrations of totally activated Protein C. Samples containing fully activated Protein C were generated by mixing Protein C at 0 to 1.5  $\mu$ M with 60 nM rabbit TM and 30 nM thrombin, incubating for 0 to 4 hours, adding  
20       hirudin and measuring S2266 activity as above. Conditions under which the Protein C was fully activated were defined as those in which the S2266 activity (A405/min) reached a plateau.

25       A unit of activity is defined as 1 pmole of activated Protein C generated per ml/min under the reagent conditions defined above. Alternatively, activity values are reported in comparison to native detergent solubilized rabbit thrombomodulin or another thrombomodulin standard.

30       ii. Protein C Cofactor Activity  
          After Exposure to Oxidants

35       Chloramine-T (N-Chloro-p-toluenesulfonamide sodium salt, Sigma) was used to specifically test the resistance of the mutant TM analog peptides to oxidation. Transfection culture supernatant (1 ml) containing a peptide encoded by a mutant TM gene sequence or pTMHY101 (wild-type, aa 227-462) was desalted into 1.5 ml of 0.2% N-ethylmorpholine (NEM), pH 7.0, 0.008% Tween 80 on a

NAP-10 column (LKB/Pharmacia) and then lyophilized and resuspended in 100  $\mu$ l of the above buffer. The sample was divided equally and either 5  $\mu$ l of water (control) of 5  $\mu$ l of 0.1 M chloramine-T (final conc.=9.1 mM) was added. The samples were incubated at room temperature for 20 minutes, then passed over the NAP-5 column to remove any oxidant. The desalting buffer used was protein C assay diluent. The mutant peptide retained all of its activity after being exposed to chloramine-T whereas the wild type peptide was substantially inactivated.

iii. Inhibition of the Activated Partial Thromboplastin Time (APTT)

The formation of a clot from citrated plasma is triggered by the addition of brain cephalin in ellagic acid ("APTT reagent"), and calcium ion. The time required for the clot to form is reproducible and increases proportionally with the addition of thrombomodulin. Reagents for the APTT are incubated at 37°C before mixing, except for the citrated plasma, which is kept at 4°C.

The reaction was carried out as follows: 100  $\mu$ l of Sigma Citrated Plasma was added to a plastic cuvette (Sarstedt #67.742), incubated at 37°C for 1 min; 100  $\mu$ l of Sigma APTT reagent was added and the mixture incubated for 2 min at 37°C; 100  $\mu$ l of test sample (or control buffer) and 100  $\mu$ l of 25 mM  $\text{CaCl}_2$  were added and the cuvette was immediately placed in a Hewlett-Packard 8451A spectrophotometer equipped with a Haake KT2 circulating water bath to keep the cuvette at 37°C during reading. The absorbance due to light scattering at 320 nm was measured every 0.5 seconds, from 15 to 120 seconds, timed from the addition of  $\text{CaCl}_2$ . A plot of absorbance vs. time yields a sigmoidal curve, with the clotting time defined as the time at which the slope is the steepest, corresponding to the inflection point of the curve.

Ex vivo APTT assays were performed in the manner described above with the exception that citrated plasma from the animal used in the *in vivo* experiment was used in place of the citrated plasma obtained commercially.

Alternatively, an APC or TCT assay can be performed as described.

iv. Inhibition of thrombin clotting time (TCT) and prothrombin reaction (PT)

Both the PT and TCT are determined using the Hewlett-Packard 8452A diode-array spectrophotometer or an equivalent used for the APTT. For the PT reaction, 90  $\mu$ l of either TM analog 6h/227-462 or PBS was added to 20  $\mu$ l thromboplastin and 90  $\mu$ l 25 mM  $\text{CaCl}_2$  in a cuvette. The mixture was incubated for 1 minute at 37°C, then 100  $\mu$ l of citrated plasma was added. After loading the cuvette into the spectrophotometer, the absorbance due to light scattering at 320 nm was measured every 0.5 seconds, from 15 to 120 seconds, timed from the addition of the plasma. A plot of absorbance vs. time yields a sigmoidal curve, with the clotting time defined as the time at which the slope is the steepest, corresponding to the inflection point of the curve. The TCT is evaluated in the same manner. The initial reaction mixture contains 100  $\mu$ l citrated plasma, 25  $\mu$ l of 100 mM  $\text{CaCl}_2$ , and 162.5  $\mu$ l of either PBS or TM analog. After 1 minute, 12.5  $\mu$ l of thrombin is added. The clotting time is measured as described above.

v. Direct anticoagulant activity - Inhibition of thrombin catalyzed conversion of fibrinogen to fibrin

Thrombin and varying amounts of TM analog 6h/227-462 were incubated for 2 minutes at 37°C in microtiter plate wells. The total initial reaction volume was 50  $\mu$ l PBS plus 7.5 mM  $\text{CaCl}_2$ , and 90 nM thrombin. After initial incubation, 100  $\mu$ l of 3.75 mg/ml human fibrinogen was added per well, and the thrombin induced formation of

fibrin was followed by measuring the change in absorbance at 405 nm in a Molecular Devices Vmax spectrophotometer (Molecular Devices, Menlo Park, CA). The end-point of the assay was the time at which 50% of the final absorbance was reached. Residual thrombin activity was determined by reference to a thrombin standard curve, which linearly relates the reciprocal of the thrombin concentration to the clotting time. When amounts of detergent solubilized native rabbit thrombomodulin and TM analog 6h/227-462 exhibiting equal activity as measured by protein C cofactor activity are compared in the direct anticoagulant activity assay, the TM analog exhibits a significantly reduced ability to inhibit thrombin-mediated conversion of fibrinogen to fibrin (approximately 1/10).

vi. Inhibition of platelet activation and aggregation

The effects of TM analog 6h/227-462 on thrombin activation of platelets was tested by the methods of Esmon, et al., (1983) J. Biol. Chem. 258:12238-12242. When evaluated using this assay, TM analog 6h/227-462 did not significantly inhibit the thrombin-mediated activation and aggregation of platelets.

viii. Additional measures of TM antithrombotic activity

1) TM analog's inhibition of activation of Factor V by thrombin is measured by the method described by Esmon et al., J. Biol. Chem., (1982), 257:7944-7947.

2) Inhibition of the TM analog thrombin complex by antithrombin III and heparin cofactor II is measured as described by Jakubowski et al. (1986), supra.

3) TM analog's inhibition of the inactivation of protein S by thrombin is measured by the method described by Thompson & Salem, J. Clin. Invest. (1986) 78(1):13-17.

4) Inhibition of thrombin-mediated activation of Factor XIII is measured by the method of Polgar, et al., (1987) Thromb. Haemostas. 58:140.



**Example 2:** *In vivo* Activity of a TM Analog in a Rodent Model of Deep Venous Thrombosis

The ability of a TM analog to abrogate the formation of a thrombus was evaluated in a modified stasis/endothelial injury-induced venous thrombosis model in the rat (see Maggi, A. et al., (1987) Haemostasis 17:329-335 or Pescador, R. et al., (1989) Thrombosis Research 53:197-201). The vena cava of an anaesthetized male Sprague Dawley rat (450 gr) was surgically isolated, then the animal was treated by bolus injection into the femoral artery with a thrombomodulin analog (6h/227-462 which contains the 6 EGF-like domains of native thrombomodulin), standard heparin or normal saline (0.1 ml/rat), as a control. The dose of heparin was 45 units/rat. The dose of thrombomodulin analog was 100, 10, 1, 0.1 or 0.01 µg/rat. Two minutes post-injection, the inferior vena cava was ligated at the left renal vein to induce stasis, and the vascular endothelium was injured by gently pinching with forceps. After 10 minutes, the vena cava was excised and examined for the presence of a thrombus, which if present was removed and weighed. In all cases the animals treated with heparin or thrombomodulin analog (6h/227-462) at 100, 10, or 1 µg/rat showed no evidence of thrombus formation whereas the saline treated animals and those receiving the lowest dose of thrombomodulin analog (0.01 µg) had thrombi with an average weight of 14.9 mg/thrombus. The rats treated with 0.1 µg of thrombomodulin analog showed a trace amount of thrombus which was not large enough to be removed and weighed.

The dose range used in this study was selected based on an *in vitro* APTT assay in which 1 µg/ml of thrombomodulin analog was insufficient to prolong the APTT but the addition of 10 µg/ml resulted in a significant prolongation. The results of APTT assays done on plasma samples taken from each of the treated rats show no prolongation in the TM analog treated and control rats (100 µg TM analog = 45 sec, all other doses TM analog and the saline

controls = 30-35 sec). However, the APTT in the heparin treated rats was significantly prolonged (100 sec).

This experimental system is a directly comparable model for deep venous thrombosis in humans, which is characterized by vascular injury and reduced blood flow. The results described above demonstrate that very low doses of a TM analog that are able to act as a cofactor for thrombin-mediated activation of protein C yet have a substantially reduced ability to inhibit thrombin-mediated conversion of fibrinogen to fibrin are effective at preventing thrombus formation. Moreover, the absence of prolongation in the APTT measured *ex vivo* indicates that this TM analog has no systemic effect on coagulation parameters and, therefore, would not promote unsafe bleeding side effects.

**Example 3: In vivo Activity of a TM Analog in a Primate Model of Both Venous and Arterial Thrombosis**

The antithrombotic properties of the thrombomodulin analogs were evaluated in an arteriovenous shunt model in the baboon using a slight modification of the method of Cadroy, Y. et al., (1989) Journal of Laboratory and Clinical Medicine 113:436-448, as described in Hanson S.R. and Harker, L.A. (1987) Thrombosis and Haemostasis 58:801-805. This model was chosen because of the hemostatic similarity between the baboon and man and because the arteriovenous shunt serves as a model for both arterial-type and venous-type thrombi.

A silastic tubing shunt, modified with a piece of dacron tubing (3.2 mm in diameter) followed by a teflon chamber (9.3 mm in diameter), was inserted into the femoral artery of the baboon such that blood flowed out of the artery through the shunt and returned to the baboon via the femoral vein (see Figure 2 of U.S. Patent Application Serial No. 07/568,456). The dacron tubing presents a thrombogenic surface which stimulates the natural coagulation process, and in particular the deposition of platelets on the graft surface, and serves

as a model for the generation of arterial, i.e. platelet rich, thrombi held together by fibrin. The chamber creates a stasis condition similar to that found in veins, where the rate of flow of the blood is reduced, and in particular mimics the area around venous valves, thus modeling flow conditions similar to those resulting in deep venous thrombosis. The thrombi formed in the chambers are venous-type, fibrin rich thrombi. Venous-type thrombi also contain platelets, but fewer than arterial-type thrombi. Thrombus formation in either the dacron graft or chamber is evaluated by measuring both platelet deposition and fibrin accretion. Platelet deposition is measured by removing platelets from the baboon, radiolabeling the platelets with <sup>111</sup>indium-oxine using the method of Cadroy, Y., et al., (1989) Journal of Clinical and Laboratory Medicine 113(4):436-448, and then returning them to the animal. A scintillation camera, such as a Picker DC 4/11 Dyna scintillation camera (Picker Corp., Northford, Conn.), is positioned over the graft to directly measure the amount of radioactivity from the platelets being deposited as part of a thrombus as described in Cadroy, Y., et al., supra. As a second measure of thrombus formation, a 5  $\mu$ Ci dose of <sup>125</sup>I-labeled baboon fibrinogen is given intravenously prior to insertion of the shunt. At the conclusion of the experiment, the shunt is removed, washed and stored for 30 days to allow for the decay of <sup>111</sup>indium radioactivity (half-life, 2.8 days). As <sup>111</sup>indium decays much more rapidly than <sup>125</sup>iodine, the detectable radioactivity remaining in the shunt represents the amount of fibrin deposited as part of a thrombus. Total fibrin deposition is calculated by dividing the counts per minute deposited by the amount of clottable fibrinogen present in the baboon blood as measured by the TCT assay. The first shunt in the series acts as a control for the second shunt.

Two shunts in series were inserted into a baboon and the TM analog (6h/227-462, see Table 4) infused at a point between the two shunts at a rate of 7 or 8 mg/hr for one hour. As can be seen in Figure 3 of U.S. Patent Application Serial No. 07/568,456, platelets were deposited in both the chamber and the dacron graft in the control shunt, however, platelet deposition was significantly reduced following infusion of the TM analog into the second shunt.

These experiments demonstrate that a TM analog that has the ability to act as a cofactor for thrombin-mediated protein C activation and has a significantly reduced ability to inhibit thrombin-mediated conversion of fibrinogen to fibrin and thrombin-mediated activation and aggregation of platelets can prevent the formation of either arterial-type or venous-type thrombi in an in vivo model. Such a TM analog would therefore be useful for pharmaceutical treatment of any thrombotic disease, whether localized to the arteries or to the veins.

**Example 4: In Vivo Circulating Half-life**

The circulating half-life of several TM analogs was evaluated using a modification of the protocol of Bakhit, C., et al., (1988) Fibrinolysis 2:31-36. Thrombomodulin analog was radiolabeled with <sup>125</sup>Iodine according to the lactoperoxidase method of Spencer, S.A., et al., (1988) J. Biol. Chem. 263:7862-7867. Approximately 100,000 cpm amount of labeled analog was injected into the femoral vein of an anesthetized mouse and small samples collected at selected time intervals. The level of radioactivity present in each sample, corresponding to the amount of radiolabeled thrombomodulin analog present in the circulation, was determined by counting in a gamma counter (Beckman) and the time necessary to decrease the amount of radioactivity in the circulation to one-half of its original value determined. These can also be based upon an APC assay and ELISA determination

Three thrombomodulin analogs were evaluated using this method: 6h/227-462 (see above), 6h/227-462 that had been pretreated with hydrofluoric acid (HF) to remove some or all of the carbohydrate, and 4t/227-462 (See Table 4 and Example 1.B.2). The treatment was done according to the method of Mort, A.J. and Lamport, T.A. (1977) Analytical Biochemistry 82:289-309. Briefly, 0.8 mg of TM analog (6h/227-462) was incubated in 1 ml anisole + 10 mls HF (conc) at 0°C for 1 hour under vacuum. After this time the volatile liquid was evaporated and the protein residue rinsed from the reaction chamber with two, 3 ml washes of 0.1 M acetic acid followed by two 3 ml washes of 50% acetic acid. The combined washes were extracted with 2 mls of ethylether to remove any residual anisole. The peptide containing aqueous phase was desalted on a PD10 column with 92% of the protein recovered from the starting material.

As can be seen from the results in Table 6, treating the TM analog so as to modify glycosylation can significantly alter its circulating half-life. This can be accomplished by either removing carbohydrate or altering its composition by expression in different cell types.

Table 1

	GGCACG GCGCAGCGGC AAGAAGTGTC TGGGCTGGGA CGGACAGGA	46
	CGGACAGGAG AGGCTGTGCG CATCGGCGTC CTGTGCCCCT CTGCTCCGGC	96
	ACGGCCCTGT CGCAGTGCCC GCGCTTTCCC CGGCGCCTGC ACGCGGCGCG	146
5	CCTGGGTAAC ATG CTT GGG GTC CTG GTC CTT GGC GCG CTG GCC Met Leu Gly Val Leu Val Leu Gly Ala Leu Ala	189
	-15 -10	
10	CTG GCC GGC CTG GGG TTC CCC GCA CCC GCA GAG CCG CAG CCG Leu Ala Gly Leu Gly Phe Pro Ala Pro Ala Glu Pro Gln Pro	231
	-5 -1 +1 5	
	GGT GGC AGC CAG TGC GTC GAG CAC GAC TGC TTC GCG CTC TAC Gly Gly Ser Gln Cys Val Glu His Asp Cys Phe Ala Leu Tyr	273
	10 15 20	
15	CCG GGC CCC GCG ACC TTC CTC AAT GCC AGT CAG ATC TGC GAC Pro Gly Pro Ala Thr Phe Leu Asn Ala Ser Gln Ile Cys Asp	315
	25 30 35	
	GGA CTG CGG GGC CAC CTA ATG ACA GTG CGC TCC TCG GTG GCT Gly Leu Arg Gly His Leu Met Thr Val Arg Ser Ser Val Ala	357
	40 45	
20	GCC GAT GTC ATT TCC TTG CTA CTG AAC GGC GAC GGC GGC GTT Ala Asp Val Ile Ser Leu Leu Leu Asn Gly Asp Gly Gly Val	399
	50 55 60	
25	GGC CGC CGG CGC CTC TGG ATC GGC CTG CAG CTG CCA CCC GGC Gly Arg Arg Arg Leu Trp Ile Gly Leu Gln Leu Pro Pro Gly	441
	65 70 75	
	TGC GGC GAC CCC AAG CGC CTC GGG CCC CTG CGC GGC TTC CAG Cys Gly Asp Pro Lys Arg Leu Gly Pro Leu Arg Gly Phe Gln	483
	80 85 90	
30	TGG GTT ACG GGA GAC AAC AAC ACC AGC TAT AGC AGG TGG GCA Trp Val Thr Gly Asp Asn Asn Thr Ser Tyr Ser Arg Trp Ala	525
	95 100 105	
	CGG CTC GAC CTC AAT GGG GCT CCC CTC TGC GGC CCG TTG TGC Arg Leu Asp Leu Asn Gly Ala Pro Leu Cys Gly Pro Leu Cys	567
	110 115	

Table 1 - (Continued)

	GTC Val 120	GCT Ala	GTC Val	TCC Ser	GCT Ala	GCT Ala 125	GAG Glu	GCC Ala	ACT Thr	GTG Val	CCC Pro 130	AGC Ser	GAG Glu	CCG Pro	609
5	ATC Ile 135	TGG Trp	GAG Glu	GAG Glu	CAG Gln	CAG Gln 140	TGC Cys	GAA Glu	GTG Val	AAG Lys	GCC Ala 145	GAT Asp	GGC Gly	TTC Phe	651
10	CTC Leu 150	TGC Cys	GAG Glu	TTC Phe	CAC His	TTC Phe	CCA Pro	GCC Ala 155	ACC Thr	TGC Cys	AGG Arg	CCA Pro	CTG Leu 160	GCT Ala	693
	GTG Val	GAG Glu	CCC Pro	GGC Gly 165	GCC Ala	GCG Ala	GCT Ala	GCC Ala 170	GCC Ala	GTG Val	TCG Ser	ATC Ile	ACC Thr	TAC Tyr 175	735
15	GGC Gly	ACC Thr	CCG Pro	TTC Phe 180	GCG Ala	GCC Ala	CGC Arg	GGA Gly	GCG Ala	GAC Asp 185	TTC Phe	CAG Gln	GCG Ala	CTG Leu	777
	CCG Pro 190	GTG Val	GGC Gly	AGC Ser	TCC Ser	GCC Ala 195	GCG Ala	GTG Val	GCT Ala	CCC Pro	CTC Leu 200	GGC Gly	TTA Leu	CAG Gln	819
20	CTA Leu 205	ATG Met	TGC Cys	ACC Thr	GCG Ala	CCG Pro	CCC Pro 210	GGA Gly	GCG Ala	GTG Val	CAG Gln	GGG Gly 215	CAC His	TGG Trp	861
25	GCC Ala	AGG Arg	GAG Glu 220	GCG Ala	CCG Pro	GGC Gly	GCT Ala	TGG Trp 225	GAC Asp	TGC Cys	AGC Ser	GTG Val	GAG Glu 230	AAC Asn	903
	GGC Gly	GGC Gly	TGC Cys	GAG Glu 235	CAC His	GCG Ala	TGC Cys	AAT Asn 240	GCG Ala	ATC Ile	CCT Pro	GGG Gly	GCT Ala	CCC Pro 245	945
30	CGC Arg	TGC Cys	CAG Gln	TGC Cys	CCA Pro 250	GCC Ala	GGC Gly	GCC Ala	GCC Ala	CTG Leu 255	CAG Gln	GCA Ala	GAC Asp	GGG Gly	987
	CGC Arg 260	TCC Ser	TGC Cys	ACC Thr	GCA Ala	TCC Ser 265	GCG Ala	ACG Thr	CAG Gln	TCC Ser	TGC Cys 270	AAC Asn	GAC Asp	CTC Leu	1029

Table 1 - (Continued)

	TGC	GAG	CAC	TTC	TGC	GTT	CCC	AAC	CCC	GAC	CAG	CCG	GGC	TCC	1071
	Cys	Glu	His	Phe	Cys	Val	Pro	Asn	Pro	Asp	Gln	Pro	Gly	Ser	
	275						280					285			
5	TAC	TCG	TGC	ATG	TGC	GAG	ACC	GGC	TAC	CGG	CTG	GCG	GCC	GAC	1113
	Tyr	Ser	Cys	Met	Cys	Glu	Thr	Gly	Tyr	Arg	Leu	Ala	Ala	Asp	
			290					295					300		
	CAA	CAC	CGG	TGC	GAG	GAC	GTG	GAT	GAC	TGC	ATA	CTG	GAG	CCC	1155
10	Gln	His	Arg	Cys	Glu	Asp	Val	Asp	Asp	Cys	Ile	Leu	Glu	Pro	
				305					310					315	
	AGT	CCG	TGT	CCG	CAG	CGC	TGT	GAG	GTC	AAC	ACA	CAG	GGT	GGC	1197
	Ser	Pro	Cys	Pro	Gln	Arg	Cys	Val	Asn	Thr	Gln	Gly	Gly	Phe	
					320					325					
15	TTC	GAG	TGC	CAC	TGC	TAC	CCT	AAC	TAC	GAC	CTG	GTG	GAC	GGC	1239
	Glu	Cys	His	Cys	Tyr	Pro	Asn	Tyr	Asp	Leu	Val	Asp	Gly	Glu	
	330					335					340				
	TGT	GTG	GAG	CCC	GTG	GAC	CCG	TGC	TTC	AGA	GCC	AAC	TGC	GAG	1281
	Cys	Val	Glu	Pro	Val	Asp	Pro	Cys	Phe	Arg	Ala	Asn	Cys	Glu	
		345					350					355			
20	TAC	CAG	TGC	CAG	CCC	CTG	AAC	CAA	ACT	AGC	TAC	CTC	TGC	GTC	1323
	Tyr	Gln	Cys	Gln	Pro	Leu	Asn	Gln	Thr	Ser	Tyr	Leu	Cys	Val	
				360				365					370		
	TGC	GCC	GAG	GGC	TTC	GCG	CCC	ATT	CCC	CAC	GAG	CCG	CAC	AGG	1365
25	Cys	Ala	Glu	Gly	Phe	Ala	Pro	Ile	Pro	His	Glu	Pro	His	Arg	
				375					380					385	
	TGC	CAG	ATG	TTT	TGC	AAC	CAG	ACT	GCC	TGT	CCA	GCC	GAC	TGC	1405
	Cys	Gln	Met	Phe	Cys	Asn	Gln	Thr	Ala	Cys	Pro	Ala	Asp	Cys	
					390					395					
30	GAC	CCC	AAC	ACC	CAG	GCT	AGC	TGT	GAG	TGC	CCT	GAA	GGC	TAC	1449
	Asp	Pro	Asn	Thr	Gln	Ala	Ser	Cys	Glu	Cys	Pro	Glu	Gly	Tyr	
	400					405					410				
	ATC	CTG	GAC	GAC	GGT	TTC	ATC	TGC	ACG	GAC	ATC	GAC	GAG	TGC	1491
	Ile	Leu	Asp	Asp	Gly	Phe	Ile	Cys	Thr	Asp	Ile	Asp	Glu	Cys	
		415					420					425			



Table 1 - (Continued)

[illegible]

	t-PA Signal Sequence														
	ATG	GAT	GCA	ATG	AAG	AGA	GGG	CTC	TGC	TGT	GTG	CTG	CTG	CTG	
5	Met	Asp	Ala	Met	Lys	Arg	Gly	Leu	Cys	Cys	Val	Leu	Leu	Leu	
			-30					-25					-20		
	TGT	GGA	GCA	GTC	TTC	GTT	TCG	CCC	AGC	CAG	INTRON A		GAA	ATC	
	Cys	Gly	Ala	Val	Phe	Val	Ser	Pro	Ser	Glu			Glu	Ile	
			-15						-10						
0	CAT	GCC	CGA	TTC	AGA	AGA	GGA	GCC	AGA	TCC					
	His	Ala	Arg	Phe	Arg	Arg	Gly	Ala	Arg	Ser					
	-5					-1	+1								

15. COD #1198 GATCATG CTC AAG TTT GTT ATT TTA TTG TGC AGT ATT  
Met Leu Lys Phe Val Ile Leu Leu Cys Ser Ile  
-15 -10  
GCC TAT GTT TTC GGT GCC GTC GTA CCA AGA TCT CCC CGG  
Ala Tyr Val Phe Gly Ala Val Val Pro Arg Ser Pro Arg  
-5 -1 +1

COD #1199

Table 3

## COD #1292

5' ATCGGATCC TGC GAA AAC GGC GGC TCC primer/coding sequence  
 5 BamHI Cys Glu Asn Gly Gly Phe  
 aa 427

## COD #1293

10 5' GTGGGATCC TGC TTC AGA GCC AAC TGC primer/coding sequence  
 BamHI Cys Phe Arg Ala Asn Cys  
 aa 350

## COD # 1294

15 5' CAGGGATCC TGC ACC CAG ACT GCC TGT primer/coding sequence  
 BamHI Cys Asn Gln Thr Ala Cys  
 aa 390

## COD #1408

20 5' (CTG GTG GAC GGC GAG TGT) coding sequence  
 GAC CAC CTG CCG CTC ACA CACCGCCGGC GCCT primer sequence  
 Leu Val Asp Gly Glu Cys NotI  
 aa 339

## COD #1409

25 5' (CGC CAC ATT GGC ACC GAC TGT) coding sequence  
 GCG GTG TAA CCG TGG CTG ACA TCTCGCCGGC GTAG primer  
 Arg His Ile Gly Thr Asp Cys NotI sequence  
 aa 456

Table 3 - (Continued)

	COD #1410	
	5' (CAC GAG CCG CAC GGA CGT) coding sequence	
5	GTG CTC GGC GTG TCC ACG GTCTCGCCGG CGTT primer sequence	
	His Glu Pro His Arg C	NotI
	aa 381	
	COD #1411	
	5' (CGC CAC ATT GGC ACC GAC TGT TGA) coding sequence	
10	GCG GTG TAA CCG TGG CTG ACA ACT CGCCGGCGT primer	
	Arg His Ile Gly Thr Asp Cys STOP	NotI sequence
	aa 456	
	COD #1412	
	5' (GAC GAC GGT TTC ATC TGC) coding sequence	
15	CTG CTG CCA AAA GGA TAC GCGCGGCCGG CTG primer sequence	
	Asp Asp Gly Phe Ile Cys	NotI
	aa 416	
	COD #1433	
	5' (CTG GTG GAC GGC GAG TGT TGA) coding sequence	
20	GAC CAC CTG CCG CTC ACA ATC CGCCGGCGCC T primer	
	Leu Val Asp Gly Glu Cys STOP	NotI sequence
	aa 339	
	COD #1434	
	5' (CAC GAG CCG CAC GGA CGT TGA) coding sequence	
25	GTG CTC GGC GTG TCC ACG ATC CGCCGGCGTT primer sequence	
	His Glu Pro His Arg Cys STOP	NotI
	aa 381	

Table 3 - (Continued)

## COD #1435

5' (GAC GAC GGT TTC ATC TGC TGA) coding sequence  
 5 CTG CTG CCA AAG GAT ACG ATC CGCCGGCGGCTG primer  
 Asp Asp Gly Phe Ile Cys STOP NotI sequence  
 aa 416

## COD #1480

5' (TGT GAC TCC GGC AAG GTG GAC TGA) coding sequence  
 10 ACA CTG AGG CCG TTC CAC CTG ACT CTTAAGCT primer  
 Cys Asp Ser Gly Cys Val Asp STOP EcoRI sequence  
 aa 462

## COD #1479

5' (GGC ACC GAC TGT GAC TCC TGA) coding sequence  
 15 CCG TGG CTG ACA CTG AGG ACT CTTAAGCAG  
 Gly Thr Asp Cys Asp Ser STOP EcoRI  
 aa 459

## COD #1478

His Trp Ala Arg Glu Ala Pro  
 20 5' CCATGGC CAC TGG GCC AGC GAG GCG CCG primer/coding  
 BalI His Trp Ala Arg Glu Ala Pro Sequence  
 aa 216

## COD #1481

5' (CCG GCC GTG GGG CTC GTG CAT TCG TGA) coding sequence  
 25 GGC CGG CAC CCC GAG CAC GTA AGC ACT CGCCGGCGGT A primer  
 Pro Ala Val Gly Leu Val His Ser STOP NotI seq.  
 aa 490

Table 4  
Expression in Insect Cells

	<u>Vector</u>	<u>TM a.a. Region</u>	<u>Domain</u>
5	pTMHY101	aa 221-462	EGFs 1-6
	pTMHY102	aa 216-468	EGFs 1-6
	pTMHY103	aa 216-464	EGFs 1-6
	pTHR10	aa 227-462	EGFs 1-6
	pTHR11	aa 227-462:227-462	EGFs 1-6 + EGfs 1-6
10	pTHR22	aa 350-462	EGFs 4,5&6
	pTHR78	aa 227-497	EGFs 1-6 + O-linked glycosylation domain
	pTHR13	aa 227-462	EGFs 1-6

Expression in Mammalian Cells

	<u>Vector</u>	<u>TM a.a. Region</u>	<u>Domain</u>
15	pTHR13	aa 227-462	EGFs 1-6
	pTHR19	aa 350-462	EGFs 4,5&6
	pTHR20	aa 227-462:227-462	EGFs 1-6 + EGfs 1-6
	pTHR21	aa 227-497	EGFs 1-6 + O-linked glycosylation
20	domain		

Table 5

Primers for replacing the Methionine at aa 291

Native Sequence

	Pro	Asp	Gln	Pro	Gly	Ser	Tyr	Ser	Cys	Met
5	CCCC	GAC	CAG	CCG	GGC	TCC	TAC	TCG	TGC	ATG
	CCCC	GAC	CAG	CCG	GGC	TCC	TAC	<u>AGC</u>	TGC	<u>CTG</u>
	Mutant Primer 1580									
										Leu
										<u>CAG</u>
	Mutant Primer 1581									
10	CCCC	GAC	CAG	CCG	GGC	TCC	TAC	TCG	TGC	<u>GCA</u>
	Mutant Primer 1582									
										Ala

	Cys	Glu	Thr	Gly	Tyr	Arg	Leu	Ala	Ala
15	TGC	GAG	ACC	GGC	TAC	CGG	CTG	GCG	GCC G
	TGC	GAG	ACC	GGC	TAC	CGG	CTG	GCG	GCC G
	TGC	GAG	<u>ACT</u>	GGC	TAC	CGG	CTG	GCG	GCC G
	TGC	GAG	ACC	GGC	TAC	CGG	CTG	GCG	GCC G

Primers for replacing the Methionine at aa 388

Native Sequence

20	Pro	His	Glu	Pro	His	Arg	Cys	Gln	Met	
	CCC	CAC	GAG	CCG	CAC	AGG	TGC	CAG	ATG	
	CCC	CAC	GAG	CCG	CAC	AGG	TGC	CAG	<u>CTG</u>	
	Mutant Primer 1573									
									Leu	
	CCC	CAC	GAG	CCG	CAC	AGG	TGT	<u>CAA</u>	<u>CAG</u>	
25	Mutant Primer 1583									
									Gln	
	CCC	CAC	GAG	CCG	CAC	AGG	TGC	CAG	<u>GCC</u>	
	Mutant Primer 1584									
									Ala	

30	Phe	Cys	Asn	Gln	Thr	Ala	Cys	Pro	Ala
	TTT	TGC	AAC	CAG	ACT	GCC	TGT	CCA	GCC G
	TTT	TGC	AAC	CAG	ACT	GCC	TGT	CCA	GCC G
	TTT	TGC	AAC	CAG	ACT	GCC	TGT	CCA	GCC G
	TTT	TGC	AAC	CAG	ACT	GCC	TGT	CCA	GCC G

Table 6.

<u>Sample</u>	<u>Half-life (min)</u>
6h/227-462	2.7
HF treated 6h/227-462	7.3
4t/227-462	8.1



**Example 5: Expression of Recombinant Thrombomodulin Genes in CHO Cells**

The cell line CHODXB11 was obtained from Larry Chasin of Columbia University. Cells were grown in HAM's F-12 complete medium (GIBCO), supplemented with 9% fetal bovine serum (FBS) and gentamicin.

**A. Transfection**

Cells,  $1 \times 10^5$ , were plated in a 100 mm petri dish one day before transfection. A plasmid pTHR525 encoding a soluble TM analog was prepared as described below in Example 9. The gene was constructed to encode an analog of thrombomodulin with the following modifications:  $\delta 3$  (truncated 3 amino acids from the natural amino terminus), Met388Leu (replacement of the methionine at position number 388 with leucine), Arg456Gly (replacement of arginine at position 456 with glycine), His457Gln (replacement of histidine at position 457 with glutamine), Ser474Ala (replacement of serine at position 474 with alanine), and  $\delta 7$  (truncated 7 amino acids from the carboxy terminus of a soluble TM at 497, i.e., the analog would end at amino acid 490). For each dish, the pTHR525 DNA to be transfected (20  $\mu$ g dissolved in 100  $\mu$ l of Opti-MEM (BRL), supplemented with mercaptoethanol (1:1000 dilution)) was mixed with a solution of Lipofectin (CalBiochem, 60  $\mu$ g in 100  $\mu$ l of Opti-MEM). The mixture was allowed to stand for 15 minutes at room temperature. Prior to the addition of the DNA mixture, the cells were washed twice with Opti-MEM and 3 ml of Opti-MEM was placed in each dish. The DNA and Lipofectin mixture was added to the plated cells and incubated overnight. The media was then changed to HAM's F-12 selective media (w/o glycine, hypoxanthine, and thymidine) and the cells allowed to recover overnight. The media was then changed to HAM's F-12 complete media with hygromycin (150  $\mu$ g/ml, CalBiochem) and the cells maintained in this media for 3 days. At the end of this

time, the media was changed to HAM's F-12 selective media (without glycine, hypoxanthine, and thymidine) supplemented with 9% dialyzed FBS and gentamicin. Clones were allowed to generate (approximately seven to ten days) and the mixed population was assayed by APC and ELISA assay. The cell population was expanded in culture flasks (225 cm<sup>2</sup>) for production purposes.

**B. Production: (1 L spinner culture x 2)**

The cultures were started with 3 g Cytodex 3 microcarrier beads (Pharmacia), 500 ml HAM's F-12 complete medium supplemented with 5% FBS, and  $8.4 \times 10^7$  total cells. The following day, media was added to bring the volume to 1 L. The supernatant was harvested every other day for about 6 weeks. 450 ml was harvested the first two times and 1500 ml on all subsequent occasions. The total harvest was 11.35 L and contained  $4.14 \times 10^6$  U.

**Example 6: Purification of TM Analogs from Cultured Supernatants**

**A. Purification of TM<sub>LEO</sub> (CHO)**

Media containing the TM<sub>LEO</sub> (CHO) analog with chondroitin or without sulfate expressed and secreted by CHO cells was made up to 0.01% Tween 80, filtered (1.2  $\mu$ M Serum Capsule #12168 and 0.2  $\mu$ M Culture Capsule #12140, Gelman Sciences, Ann Arbor, MI), loaded on a 100 ml Q-Sepharose column, washed with 50 mM Tris-HCl pH 7.8, 0.2 M NaCl, 0.1 mM EDTA, 0.01% Tween 80, and eluted with a NaCl gradient (0.2 to 2M) in the same buffer. Peak A (TM without chondroitin sulfate) and Peak B (with chondroitin sulfate) were diluted to 0.3 M NaCl, 20 mM Tris-HCl, 0.5 mM CaCl<sub>2</sub>, EDTA, 0.02% NaN<sub>3</sub>, pH 7.5. A thrombin affinity chromatography step is performed essentially as described above, and the samples desalted. Active fractions were pooled.

**B. Purification of  $TM_2$  (Sf9).**

These products can be isolated as described above or as follows:

All procedures were performed at 4°C. Filtered insect cell harvest was diluted 1:1 with water, titrated with acetic acid to pH 5.2 and loaded on a Q-Sepharose fast flow resin (25 mM Na-acetate, pH 5, 0.1 M NaCl, 0.02%  $NaN_3$ ). Active fractions were eluted with 0.3 M NaCl in the same buffer, pooled, diluted to 0.3 M NaCl, 20 mM Tris-HCl, 0.5 mM  $CaCl_2$ , 0.02%  $NaN_3$ , adjusted to pH 7.5 (NaOH), loaded on a 120 ml DFP-inactivated thrombin Affigel-10 resin (described above) and eluted with 2 M NaCl, 20 mM Tris-HCl, 1 mM  $Na_2EDTA$ , 0.02%  $NaN_3$ , pH 7.5. Active fractions were pooled, desalted and buffer exchanged on a Sephadex G-25 column into 0.2% NEM-Ac, pH 7, loaded on a Mono-Q HR10/10 column (Pharmacia), and gradient eluted between 0 and 1 M NaCl in the same buffer. High specific activity fractions (APC assay) were pooled, desalted on Sephadex G-25 into PBS or 0.2% NEM-Ac, pH 7 and stored frozen or lyophilized.

**C. Soluble  $TM_{LEO}$  Expression in CHL1 Cells**

DNA coding for both the wild type and M388L mutant forms of TM, amino acids 1 to 497, and full length TM, amino acids 1 to 557, were expressed in Cos 7 and CHL1 cells using the mammalian expression vector pRc/CMV, obtained from Invitrogen, San Diego, California. For transient expression, Cos 7 cells expressing full length TM were harvested 48 to 72 h post transfection. CHL1 cells are a human melanoma cell line, available from the ATCC.

**D. Purification of  $TM_{LEO}$  (CHL1)**

Media containing the  $TM_{LEO}$  (CHL1) analog with chondroitin sulfate expressed and secreted by CHL1 cells was made 0.01% in Tween 80, filtered (1.2  $\mu$ M Serum Capsule #12168 and 0.2  $\mu$ M Culture Capsule #12140, Gelman

Sciences, Ann Arbor, MI), loaded on a 100 ml Q-Sepharose column, washed with 50 mM Tris-HCl pH 7.8, 0.2M NaCl, 0.1 mM EDTA, 0.01% Tween 80, and eluted with a NaCl gradient (0.2 to 2 M) in the same buffer (elutes near 1 M NaCl by APC assay). The eluent was diluted 3-fold with H<sub>2</sub>O, loaded on a second 5 ml Q-Sepharose column, washed with the same buffer except with 0.001% Tween 80 and 0.7 M NaCl and eluted with a second shallow gradient (30 column volumes; 0.7 to 1.6 M NaCl). Small amounts (<500 µg) were further purified by molecular exclusion chromatography on Superose 6 (Pharmacia) in PBS.

#### E. Anion Exchange Chromatography

As noted above, more than one form of TM<sub>2</sub> analog is detected in thrombin-affinity purified material from Sf9 cells run on a SDS-PAGE gel under non-reducing conditions. A method was developed to resolve these variants. TM<sub>2</sub> (Sf9) analog 6h/227-462, TM<sub>2</sub> (Sf9) collected from the Sephadex G25 column, as described above, was applied to a Mono Q column (Pharmacia, 10 micron particles, quaternary amine) pre-equilibrated with 0.2% N-ethylmorpholine (NEM) pH 7.0. After washing with this buffer the various forms were separated using a gradient of 0 to 0.4 M NaCl. The elution and activity profiles are shown in Figure 1. Samples of each fraction were evaluated on an SDS-PAGE gel under non-reducing conditions. Three distinct bands with slightly different mobilities could be seen on the stained gel. Fractions containing peptides with like mobilities were pooled (A = fractions 32-35, B = fractions 40-44, C = fractions 70-71) and then assayed for total protein content and for activity in the Protein C activation assay. The specific activities are listed in the table below. No inactive peptides were detectable in any of the fractions.

<u>Test Material</u>	<u>Specific Activity (U/mg)</u>
Mono Q Load	166,000 ± 12,000
fractions 32 - 35 (A)	416,000 ± 19,000
fractions 40 - 44 (B)	262,000 ± 4,000
fractions 70 - 71 (C)	67,600 ± 5,000

### B. SDS-PAGE Analysis

Every fraction or every other fraction of  $TM_2$  (Sf9) purified on the Mono-Q column was analyzed by SDS-PAGE as follows: 4.5  $\mu$ l of a fraction was mixed with 1.5  $\mu$ l of 4 x concentrated SDS-PAGE sample buffer without reducing agents, heated about 10 to 15 min. at about 90°C and loaded onto a 8-25% acrylamide Phast gel from Pharmacia using the comb and protocol provided. The gel was run on the Phast gel system using the protocol suggested by the manufacturer. The gel shows fractions across the peak and shoulder which were pooled to make Pool and B, respectively (Figure 2). The abbreviations are as follows:

Numbers - Fraction numbers referred to in Fig. 1 and above Table listing specific activities.

A - occasionally seen artifact band in load on Panel A, frac. 28 and frac. 39; this appears to be from protein from fingerprints on labware.

D -  $TM_2$  disulfide linked dimer which elutes in Pool C

MU - upper monomer, this appears to be the single-chain  $TM_2$

ML - lower monomer, has the same N-terminus as MU (AlaValValPro...), but has a faster electrophoretic mobility as could be expected of a protein proteolyzed near the C-terminus

L - material loaded on the Mono-Q column containing D, MU and ML

By pooling the fractions indicated in the above table, MU or ML can be greatly enriched, as desired.

#### Example 7: Demonstration of the Presence of Two-Chain $TM$

##### A. Separation of Cleaved Forms from CHL1 and CHO Cells

The following samples were analyzed on an 8% Tris-Glycine gel from Gel Novex, all samples were run under reducing conditions. Figure 3 shows the results for the following samples:

<u>Lane</u>	
	1 Novex Wide Range Marker
	2 TM <sub>LEO</sub> (CHO) #82891 LJ
5	3 TM <sub>LEO</sub> (CHO)PkB + Chondroitinase ABC ( <i>P. vulgaris</i> )
	4 TM <sub>LEO</sub> (CHO)PkB + Chondroitinase AC ( <i>Flavobacterium heparium</i> )
	5 TM <sub>LEO</sub> (CHL1)PkB + Chondroitinase AC ( <i>Flavobacterium heparium</i> )
10	6 TM <sub>LEO</sub> (CHO)PkB + Chondroitinase AC ( <i>Anthrobacter aureus</i> )
	7 TM <sub>LEO</sub> (CHL1)PkB + Chondroitinase AC ( <i>Flavobacterium heparium</i> )
	8 TM <sub>LEO</sub> (CHL1)PkB + Chondroitinase ABC ( <i>P. vulgaris</i> )
15	9 TM <sub>LEO</sub> (CHL1)PkB #Q617
	10 Novex Wide Range Markers

This gel was originally run to test commercial chondroitinases from various sources. It shows that samples from CHO cells and CHL1 cells both contain the 80 kDa band which appears to be the truncated form of the full length soluble TM analogs. The band at 66 kDa is BSA which is added to the commercial chondroitinase preparations for stability. Lane 9 is underloaded.

#### B. Western Blot

Samples of purified TM and TM analogs were analyzed on an 8% Tris-Glycine gel, which was run at 125 volts for 2 hours. The proteins were electroblot-transferred to nitrocellulose for 3 hours at 120 MA. The nitrocellulose was then incubated overnight with 3% BSA at 4°C. The blot was then exposed to a first mouse polyclonal antibody preparation which were raised against the reduced and denatured 6EGF region of TM, at a 1:500 dilution for 30 minutes. Then, a second antibody, a commercial biotinylated Goat was added for 30 minutes. The resulting nitrocellulose blot was then developed with avidin-HRP conjugate and 4-Chloro-T-Naphthol, from a commercial supplier.

Figure 4 shows a Western blot gel in which:

Lane

	1	10 $\mu$ l BRL Biotinylated markers
	2	TM <sub>LEO</sub> (CHL1)PkB #QG17 (~1250 APC Units)
	3	TM <sub>LEO</sub> (CHO)PkB #82891 LJ (~130 APC Units)
5	4	TM <sub>LEO</sub> (CHO)PkB #82891 LJ (~125 APC Units)
	5	TM <sub>LEO</sub> (CHL1)PkB + Chondroitinase ABC #QG21 (~106 APC Units)
	6	TM <sub>LEO</sub> (CHO)PkB + Chondroitinase ABC #QG39 (~34 APC Units)
10	7	TM <sub>LE</sub> (CHL1)PkB + Chondroitinase ABC #QG41 (~100 APC Units)
	8	5 $\mu$ l "Rainbow" markers, Amersham
	MW Markers for Fig. 4; BRL Biotinylated "Rainbow"	
		97.4 kDa      200 kDa
15		66.2      97.4
		42.7      69.0
		31.0      46.0
	Run with {	21.5      30.0
20	Dye Front {	14.4      21.5 } Run w/Dye
		14.3 } Front

This Western analysis shows that all samples of TM<sub>LEO</sub> expressed in CHO cells have an immunoreactive band at 80 kDa which appears to be the truncated form of TM and that some samples of TM<sub>LEO</sub> expressed in CHL1 cells have the 80 kDa band (Lane 2). Lane 7 containing the "lectin-6EGF" analog, TM<sub>LE</sub> is underloaded.

**C. K<sub>a</sub> Determination**

The K<sub>a</sub> was determined for isolated preparations of recombinant thrombomodulin. TM<sub>LEO</sub>(CHO) was prepared as described earlier. Determinations were made in 96 well plates in an assay diluent (20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.1% NaN<sub>3</sub>, 0.5% BSA, containing either 0.25 or 2.5 mM CaCl<sub>2</sub>). For the K<sub>a</sub> determination, thrombin (1 nM) was added to the TM analog (0.5 to 250 nM); reaction was initiated by Protein C addition (3  $\mu$ M). All concentrations listed are final concentrations. Mixtures were incubated 10-60 minutes (75  $\mu$ l, 20°C) and quenched with hirudin (570 nM). 100  $\mu$ l/well of S-2266 substrate (Kabi Vitrum), in modified assay diluent, was then added (2 mM).

As can be seen from the double reciprocal plot in Figure 5, the actual first point, corresponding to high TM concentration, is displaced from the linear projection derived from the measurements at lower TM concentrations. This indicates that another TM component with a significantly high  $K_d$  is present in the test sample. It appears that there are two classes of thrombin binding species in the sample, both of which lead to the formation of an active complex. The low affinity form is likely to correspond to the cleaved or truncated form, e.g., a TM analog which does not bind thrombin as tightly as uncleaved TM.

**Example 8: Sequencing Data Demonstrating Two Different Amino Termini**

**A. N-terminal Sequence Analysis**

The purified protein was dried and sequenced (Applied Biosystems, Model #477 with a 900A data module or Model #470A with chart recorder). PTH-amino acids were identified on a 120A Applied Biosystems PTH Analyzer by RP-HPLC (Brownlee PTH-C18 cartridge, 2.1 x 222mm).

**B. N-terminal Analysis**

Various preparations of TM analogs were analyzed as above. As can be seen in Table 7, the samples from  $TM_{LEO}$  (CHO) peak A contain heterogeneous N-termini. For example, N-terminal analysis of 3 samples shown in Table 7 of TM produced by a plasmid containing the unmodified sequence expressed in CHO cells showed a strong cleavage site sequence amounting to 5 to 19% ( $11 \pm 4\%$ ) of the protein present.

In contrast, N-terminal analysis of at least 100 picomole of DXB-11 525, a TM preparation isolated from CHO cells transformed with the pTHR525 plasmid containing the TM mutations at the indicated positions ( $\delta 3, M388L, R456G, H457Q, S474A, \delta 7$ ) confirmed that this polypeptide contained essentially no N-terminal heterogeneity (maximum second sequence is  $0.6 \pm 1.350 \approx 0\%$ ). In



particular, no secondary amino terminus corresponding to a natural sequence of :HIGT was seen, thus indicating absence of detectable protease cleavage at that, or any other, site.

5

**TABLE 7**

Sequence Comparison of Samples of TM<sub>LEO</sub>(CHO)P<sub>kA</sub> with TM<sub>LEO</sub>(CHO)03,M388L,R456G,H457Q,S474A,07

Notes:

- 1) Data is pmol of amino acid per cycle and percent clipped calculated per cycle.  
 2) Amino acids which are difficult to quantitate reliably: His, Arg, Ser, Thr  
 3) Cys not quantitated since samples not reduced and alkylated.  
 4) Pro often not well cleaved, therefore yield may be low.  
 5) Pmol data corrected for background signal and "lag" signal from previous cycles.

A) Sample: TM<sub>LEO</sub>(CHO)P<sub>kA</sub> 82891; 10/23/91

	<u>Cycle</u>	<u>N-term#1</u>	<u>N-term#2</u>	<u>Sum</u>	<u>Clip</u>	<u>%</u>
15	1	Ala 110	Phe 57	167	His 0.6	
	2	Pro -	Pro -	115	Ile 11	9.5
	3	Ala -	Ala -	127	Gly 24	18.9
	4	Glu 30	Pro 49	79	Thr 10	12.6
20	5	Pro 72	Ala 71	143	Asp 7.1	5.0
	6	Gln 41	Glu 26	67	Cys -	
	7	Pro -	Pro -	86	Asp 8.5	9.9

B) Sample: TM<sub>LEO</sub>(CHO)P<sub>kA</sub> 91991; 10/28/91

	<u>Cycle</u>	<u>N-term#1</u>	<u>N-term#2</u>	<u>Sum</u>	<u>Clip</u>	<u>%</u>
25	1	Ala 221	Phe 108	329	His 8	
	2	Pro -	Pro -	228	Ile 13	5.7
	3	Ala -	Ala -	321	Gly 37	11.5
	4	Glu 128	Pro 114	242	Thr 7	
	5	Pro 182	Ala 125	307	Asp 40	13
30	6	Gln 147	Glu 114	67	Cys -	
	7	Pro -	Pro -	328	Asp 126	

C) Sample: TM<sub>LEO</sub>(CHO)P<sub>K</sub>A 102491-A; 1/17/92

	<u>Cycle</u>	<u>N-term#1</u>	<u>N-term#2</u>	<u>Sum</u>	<u>Clip</u>	<u>%</u>
	1	Ala 142	Phe 60	202	His 0	0
	2	Pro -	Pro -	118	Ile 20	17
5	3	Ala -	Ala -	160	Gly 20	12.5
	4	Glu 48	Pro 38	86	Thr 8	9.3
	5	Pro 48	Ala 60	108	Asp 41	
	6	Gln 52	Glu 39	91	Cys -	
	7	Pro -	Pro -	58	Asp 42	

10 D) Sample: TM<sub>LEO</sub>(CHO)03,M388L,R456G,H457Q,S474A,07; pTHR525

	<u>Cycle</u>	<u>N-term</u>	<u>Clip</u>	<u>%</u>
	1	Glu 88	Gln 0	0
	2	Pro 184	Ile 0.4	0.2
	3	Gln 171	Gly 6	3.5
15	4	Pro 172	Thr 0	0
	5	Gly 149	Asp 0	0
	6	Gly 143	Cys -	
	7	Ser 28	Asp 0	0

20 Example 9: Method of Mutagenesis and Oligonucleotide Selection for Production of Protease-Resistant TM Analogs

25 The plasmid pTHR525, coding for a preferred embodiment of a protease-resistant TM, was constructed by mutagenesis of the TM gene (see U.S. Patent Application Serial No. 07/345,372, GenBank®, or the sequence of pTHR324 shown in Table 8; pTHR324 contains the TM gene in the pRC/CMV vector). A gene construct which, upon expression, expresses a polypeptide corresponding to amino acids 3-490 was made by standard mutagenesis techniques.

A. Modification of the protease cleavage site

The Arg<sup>456</sup>/His<sup>457</sup> ---> Gly<sup>456</sup>/Gln<sup>457</sup> mutation was constructed as disclosed in Example 1(C) using as a primer oligomer COD-2218.

5

B. Modification of the O-linked chondroitin sulfate linkage site

The Ser<sup>474</sup>→Ala<sup>474</sup> mutation was constructed as disclosed in Example 1(C) using as a primer oligomer COD-1886.

C. Modification of the N-terminus

10

The N-terminal deletion of the first three amino acids of native TM, which has a heterogeneous signal sequence cleavage site, was constructed as disclosed in Example 1(C) using as a primer oligomer COD-2321. The construct has a change in the fourth glycine of the signal sequence (i.e., amino acid -3) from Gly to Val, in addition to providing an N-terminal sequence, after processing of the signal sequence, which begins at amino acid Gly4. This construct was prepared based upon predictions of signal cleavage efficiencies using algorithms described in von Heijne, G., Nucleic Acids Res. 14 4683 (1986).

15

20

TABLE 8

CGGGCCGC  
tcgagcatgcatctagagggccctatt  
ctatagtgacactaaatgctcgctgatcagcctcgactgtgccttctagtggccagccatctgtgtttgccccctccc  
cgtgccttccctgacccctgg  
aagggtgccactcccactgtcctttcctaataaaatgaggaaatgcatcgcatgtcttgagttaggtgtcatctctattctg  
gggggtgggggtggggcagga  
cagcaagggggaggatgggaagacaatagcaggcatgctggggatgagggtgggctctatggaaccagctggggctcgag  
gggggatccccacggccct  
gtagcggcgcatgaagcggcggggtgtgggtgttacggcgagcgtgaccgctacacttgccagcggccctagcggccgct  
cctttcgctttcttcccttc  
ctttctcgccacgttcgcccggctttccccgtcaagctctaaatcggggcatccctttaggggtccgatttagtgctttac  
ggcacctcgaccccaaaaa  
cttgattaggggtgaggttcacgtagtgggcatcgccctgatagacgggttttcgaccttactgagcactctttaatag  
tggactcttgttccaaactg  
gaacaacactcaacccatctcggtctattcttttgatttataagatttccatcgccatgtaaaagtgttacaattagca  
ttaaattactctttatag  
ctactattcttttggtctcgttcacgggggtgggtaccgagctcgaattctgtggaatgtgtgtcagttaggggtgtggaa  
gtccccaggctccccaggca  
ggcagaagtatgcaaagcatgcatctcaattagtcagcaaccagggtgtggaaagtccccaggctccccagcaggcagaag  
tatgcaaagcatgcatctca  
attagtcagcaaccatagtcggccctaaactcgcccatcccgccctaaactcgccagcttccgcccattctccgccc  
catggctgactaatttttt  
tatttatgagagggccgagggccgctcgccctctgagctattccagaagttagtgaggaggcttttttgaggccctaggct  
tttgcaaaaagctccggga  
gcttggatattccattttcggtatctgatcaagagacaggatgaggatcgtttcgcatgattgaacaagatggattgcacgc  
agggtctcggggcgcttggg  
tggagaggctattcggtatgactgggcacacagacaaatcggtgctctgatgccggcgtgttccggctgtcagcgag  
ggggcgccgggtctttttgt  
caagaccgacctgtcgggtgcccgaatgaactgcaggacgaggcagcgggctatcgtggctggccacgacggggcgttc  
cttgcgcagctgtgctcgac  
gttgtcactgaagcgggaagggactgggtgctattgggcgaagtgcggggcaggatctcctgtcatctcaccttgcctcc  
tgccgagaaagtatccatca  
tggctgtagcaatgcccgggtgcatacgcttgatccggctacctgcccattcgaccaccaagcgaaacatcgcatcgag  
cgagcacgtactcggaatgga  
agccggtcttgcgacaggatgactcggacgaagagcatcaggggctcggcgccagccgaactgttcgccaggctcaagg  
cgcgcatgcccagcggcgag  
gatctcgtcgtgacccatggcgatgcctgcttgccgaatatcatggtggaaaaatggccgcttttctggattcatcgactg  
tggccggctgggtgtggcgg  
accgctatcaggacatagcgttggctaccgctgatattgctgaagagcttggcggcgaaatgggctgaccgcttccctcgtg  
ctttacgggtatcgccgctcc  
cgattcgcagcgcctcgtctctatcgcccttcttgacgagttcttctgagcgggactctgggggtcgaaatgaccgacca  
agcgacgcccacactgccat  
cagagatttcgattccaccgccccttctatgaaagggtggggcttcggaatcggtttccgggacgcccggctggatgac  
ctccagcgggggatctcat  
gctggagttcttcgcccaccccaacttggtttattgcagcttataatgggttacaataaagcaatagcatcacaatttca  
caataaagcattttttca  
ctgcatctagttgtgggttgcctcaaaactcatcaatgtatcttatcatgtctggatcccgctcgactcgagagcttggcg  
taatcatgggtcatagctgtt  
tccgtgtgaaattgttatccgctcacaattccacacaacatacagagccggaagcataaagtgtaaagcctgggggtgcct  
aatgagtgagctaaactcaca  
ttaattgcgttgcgtcactgcccgtttccagtcgggaaacctgtcgtgccagctgcattaatgaaatcgggccaacggcg  
ggggagagggcgtttgcgta  
tggggcgctctccgcttccctcgctcactgactcgctgcgtcggtcgttcggctgcccggagcggatcagctcactca  
aaggcggtaatacgggtatc  
cacagaaatcaggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgct  
tgctggcggttttccatagg

TABLE 8 (cont'd)

gacggatcgggagatctcccgatcccctatgggtcgactctcagtagacaatctgctctgatgccgcatagtagccagtagat  
ctgctccctgcttgtgtgtt  
ggaggtcgctgagtagtgcgcgagcaaaatttaagctacaacaaggcaaggcttgaccgacaattgcatgaagaatctgc  
ttagggttaggcgttttgcg  
ctgcttcgggatgtacgggagatatacgcgttgacattgattattgactagttattaatagtaatacaattacgggggtc  
attagttcatagcccatata  
tggagtccgcgttacataacttacggtaaatggccgcctgggtgaccgcccacgacccccgccattgacgtcaata  
atgacgtatgttcccatagt  
aacgccaatagggaactttccattgacgtcaatgggtggactatttacggtaaacgcccacttggcagtagacatcaagtgt  
atcatatgccaagtacgccc  
cctattgacgtcaatgacggtaaatggccgcctggcattatgcccagtagacattatgggactttcctacttggca  
gtacatctacgtatttagtca  
tcgctattaccatgggtgatgcgggtttggcagtagacatcaatgggcgtggatagcgggtttgactcaccggggatttccaagt  
ctccaccccatgacgtcaa  
tgggagtttgttttggcaccaaaatcaacgggactttccaaaatgtcgtaacaactccgccccattgacgcaaatggggg  
gtaggcgtgtacgggtgggag  
gtctatataagcagagctctctggctaactagagaacccactgcttaactggcttatcgaaattaatcagactcactata  
gggagaccgg  
AAGCTTCCTCGAGCGATATCGCCGCGGCATCGATCGGGCCCGAGATCTCGCGCGCTGGGTAAATGCTTGGGGTCTCTGG  
TCCTTGGCGCGCTGG  
CCCTGGCGGCGCTGGGGTTCCCCGCAACCCGAGAGCCCG  
AGCCGG  
GTGGCAGCCAGTGCCTCGAGCAGCACTGCTTCGCGCTCTACCCGGGCCCCGCGAC  
CTTCCTCAATGCCAGTCAGATCTGCGACGGAAGTCCGGGGCCACCTAATGA  
CAGTGCCTCTCTCGGTGGGTGCGGATGTCTTCCTTCTGCTACTGA  
ACGGCGAGCGGCGGCTTGGCGCGCGGCGCTCTGGATCGGCTGCAGCTGCCACCCGGCTGCGGCGACCCCAAGCGCCTC  
GGGCGCCTGCGCGGCTTCCAGTGGGTACGGGAGACAACAACACAGCTATAGCAGGTGGGCGACGGCTCGACCTCAATGG  
GGCTCCCTCTGCGGCGCGCTTGTGCGTCTGCTCCGCTGTGAGGCCACTGTGCCAGCGAGCCGATCTGGGAGGAGC  
AGCAGTGGGAAGTGAAGGCCGATGGCTTCCTCTGCGAGTTCACCTTCCAGGCACCTGCAGGCCACTGGCTGTGGAGCCC  
GGCGCGCGGCTGCCGCGCTCTCGATCACCTACGGCACCCCGTTCGCGGCGCGGAGCGGACTTCCAGGCGCTGCCGGT  
GGGCG  
CTCCGCGCGGCTGGCTCCCTCTGGCTTACAGCTAATGTGCAACGCGCGCGCGGAGCGGTCCAGGGGCACTGGGCCAGGG  
AGGCGCGGGGCGCTTGGGACTGCAGCGTGGAGAACCGCGG  
CTGCGA  
GCACGCGTG  
CAATGCCGATCCCTGGGGCTCCC  
CGCTGC  
CAGTGCCGAGCGGCGCGCGCTTGCAGGCAGAGGGGCGCTCTGCACCGCATCCGCGACGCACTCCTGCAACGACCTCTG  
CGAGCACTTCTGCGTTCCCAACCCGACAGCGGGCTCTACTCTGTCATGTGCGAGACCGGCTACCGGCTGGCG  
GGCGACCAACACCGGTGCGAGGACGTGGATGACTGCATACTGGAGCCAGTCCGTGTCCGAGCGCTGTGTCAACACACA  
GGGTGGCTTCTGAGTGCCACT  
GCTACCTTAACCTACGACCTGGTGGACGGCGAGTGTGTGGAGCCCGTGGACCCGTGCTTCAGAGCCAACTGCGAGTACCAG  
TG  
CCAGCCCCCTGAACCAAAGTACCTCTGCGTCTGCGCGAGGGCTTCCGCGCCATTCCCCACGAGCCGACAGGTGCC  
AGATGTTTTGCAACCACT  
GCCTGTCCAGCCGACTGCGACCCCAACCCAGGCTAGCTGTGAGTGCCCTGAAGGCTACATCCTGGACGACGGTTTCAT  
CTGCACGGACATCGACGAGT  
GCGAAAACGCGCGGCTTCTGCTCCGGGTGTGCCAACCTCCCCGGTACCTTCGAGTGCATCTGCGGGCCCGACTCGGCC  
CTTGGCGGCCACATTGGCAC  
CGACTGTGACTCCGGCAAGGTGGACGGTGGC  
GACAGC  
GGCTCTGGCGAGCCCCCGCCAGCCCCAGCG  
CCGGCT  
CCACCTTACTCTCCGCGCTGGGGCTGTGATTGGTGA

TABLE 8 (cont'd)

ctccgccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagatacca  
ggcggtttccccctggaagct  
ccctcgtagcgtctcctgttccgacctgcccgttacggatacctgtccgcctttctcccttcgggaagcgtggcgctt  
tctcaatgctcacgctgtag  
gtatctcagttcgggtgtaggtcgttcgctccaagctgggctgtgtgcacgaaccccccttcagccccgaccgctgcgcct  
tatccggtaactatcgtctt  
gagtcacacccggttaagacacgacttatcgccactggcagcagccacttgtaacaggattagcagagcgaggtatgtagg  
cgggtgctacagagttcttga  
agtgggtggcctaactacggctacactagaaggacagattttgggtatctgcgctctgctgaagccagttaccttcggaaaa  
agagttggtagctcttgatc  
cggcaaacacacccgctggtagcgggtgggttttttggtttgcaagcagcagattacgcgcagaaaaaaggatctcaag  
aagatcctttgatcttttct  
acggggtctgacgctcagtggaacgaaaactcacgttaagggattttgggtcatgagattatcaaaaaggatcttcaccta  
gatccttttaaaataaaaaat  
gaagttttaaatcaatctaaagtatatatgagtaaaacttggtctgacagttaccaatgcttaatcagtgaggcacctatc  
tcagcgatctgtctatttcg  
tccatccatagttggcctgactccccgctggtgtagataactacgatacgggaggggcttaccatctggccccagtgctgcaa  
tgataccgcgagacccacgc  
tcacgggtccagatttatcagcaataaaccagccagccgggaagggcgagcgcagaagtggtccctgcaactttatccgc  
ctccatccagtcctattaatt  
gttgccgggaagctagagtaagtagtccgacgttaatagtttgccgaacggttggttgccattgctacaggcatcgtggtg  
tcacgctcgtcgtttgggtat  
gggttcattcagctccggttcccaacgatcaaggcgagttacatgatccccatgtgtgcaaaaaagcgggttagctcct  
tcggtcctccgactcgttgc  
agaagtaagttggcgcagtggttatcactcatgggtatggcagcactgcataattctcttactgtcatgccatccgtaag  
atgctttctgtgactgggtg  
agtactcaaccaagtcattctgagaaatagtgatgcccgcagcaggttgctcttgccggcgctcaatacgggataatacc  
gcgccacatagcagaacttt  
aaaagtgtctcatcttggaacacgttcttcggggcgaaaactctcaaggatcttaccgctgttgagatccaggttcgatgt  
aaccacactcgtgcacccaac  
tgatcttcagcatcttttactttcaccagcgtttctgggtgagcaaaaacaggaaggcgaataatgcccgaataaagggaat  
aaggcgacacggaaatgtt  
gaatactcatactcttcttttcaatattattgaagcatttatcagggttattgtctcatgagcgggatacatatttgaa  
tgtatttagaaaaataaaca  
aataggggttcgcgcacatttccccgaaaagtccacctgacgtc  
\*HIND3-NOT1 fragment from pTHR322 containing the DNFL TM gene into the HIND3-NOT  
1 sites of pRCMV for expression of DNFL in COS1 cells.

TABLE 9

## C-Terminal Modifications

ECICGPDSALARHIGTDCDSGKYDGGDSGSGEPPPSPTPGSTLTPPAVGLVHS



ECICGPDSALAGQIGTDCDSGKYDGGDSGAGEPPPSPTPGSTLTPP

#### D. Modification of the C-terminus

The C-terminal deletion of the terminal 7 amino acids of the TM<sub>LEO</sub> polypeptide to produce a polypeptide ending at amino acid 490 of TM (with reference to the native human protein) to produce an exoprotease-resistant Pro-Pro sequence C-terminus was constructed as disclosed in Example 1(C) using as a primer oligomer COD-2320. A summary of the modifications to the C-terminus is shown in Table 9.

#### E. Summary of the Construction of pTHR525

The starting plasmid, pCDM8, for the following constructs was obtained from Invitrogen, San Diego, California. It carries the cytomegalovirus immediate early promoter. pTHR219 was cut with and the MluI-NotI fragment isolated. This fragment was inserted into pTHR211 yielding pTHR253. pTHR253 was mutagenized with COD2218 to convert R456G and H457Q, yielding pTHR491. pTHR491 was cut and the Kpn-NotI fragment isolated. This fragment was inserted into the Kpn-NotI site of pTHR470, carrying the S474A mutation yielding pTHR496. This plasmid contains the mutations S474A, R456G, and H457Q. pTHR496 was cut and the MluI-NotI fragment isolated. This fragment was inserted into the MluI-NotI site of pTHR235 yielding pTHR511. This plasmid contains the mutations S474A, R456G, H457Q, and the upstream TM sequences from the NotI site. pTHR511 was mutagenized with COD2320 to remove the 7 C-terminal amino acids yielding pTHR514. pTHR514 was cut and the MluI-NotI fragment isolated. This fragment was inserted into pTHR518 yielding pTHR524. pTHR518 was constructed by inserting the ClaI-SmaI fragment from pTHR515 into pTHR512. pTHR524 was cut and the XbaI-NotI fragment isolated. This fragment was inserted into pTHR495 at the XbaI-NotI site yielding pTHR525. pTHR525 is expressed to make one of the preferred thrombomodulin analogs having N-terminal  $\delta$ 3, R456G, H457Q, S474A, and C-terminal  $\delta$ 7.

Table 10 shows sequences from the various intermediate plasmid constructs.

Table 10

pTHR498

GAATTTTAAAGTGTGCTCATTCATTGGAAACCTTTCTTGGGGGCGAAAACTCTCAAGGATC  
TTACCGCTGTTGAGATCCAGTTCTGATGTAAACCCACTCGTGCACCCCACTGATCTTCAGC  
ATCTTTTACTTTTCACGAGCTTTCTGCGTGAAGCAAAACAGGGAAGGCAAAATGCGGAA  
AAAAGGGGAATAGGGCGACACGGAATGTGAATACTCATACTCTTCCTTTTCAATAT  
TATTGAAGCATTTATCAGGCTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTA  
GCAAAATAACAAATAGGGCTTCCGCGCAGCATTTCCCGGAAAAGTCCACCTGACGCT  
AAGAAACCATTATTATCATGACATTAACCTATAAAATAGGCGTATGACGAGGCTTT  
CCTCTTCAATTCAGGCGATTTTGAAGCTTATC  
ATCGAT

\*KPN1-NOT1 fragment from pTHR491 containing the Olink domain into the KPN1-NOT1 sites of pTHR470 to make a mammalian expression plasmid that expresses DNFL with the R456G, H457Q, and S474A mutations.

pTHR491

AGCGAAGCGAGCGAGGACTGGGCGGCGGCCAAAGCGCTCGGACAGTGTCTCCGAGAGCGGCTCGCATAGAAAT  
TGATCAACGCATATAGCGCTAGCGAGCAGCCATAGTGACTGGCGATGCTGTGGAATGGAC  
GATATC  
CCGC  
AAGAGGCGCGGCGAGTACCGGCATAACCAAGCCTATGCTACAGCATCCAGGCTGACGGTGCCGAGGATGACG  
ATGAGCGCATTGTTAGATTTATACACGGTGCCTGACTGCGTTAGCAATTTAACTGTGATAAACTACCGCAT  
TAAAGCTTATCGATGATAAGCTGTCAACATGAGAATTC  
TTG

AAGACGAAAGGGCCTCGTGATACGCCTATTTTATAGGTTAATGTCATGATAATAATGTTTCTTAGACGTC  
\*COD2218 was used to convert Arg 456 Gly and His 457 Gln by in vitro mutagenesis in the vector pTHR235. This created a MSC1 site. This creates an O-link domain that should not be cleaved in CHO cells. Also, COD1836 was used to convert Ser 474 to Ala, to eliminate a potential GAG site in the O-link domain. This created a NAR1 site. Also, COD1689 was used to convert AMP sensitive to AMP resistant.

pTHR235

AGCGAAGCGAGCGAGGACTGGGCGGCGGCCAAAGCGCTCGGACAGTGTCTCCGAGAGCGGCTCGCATAGAAAT  
TGATCAACGCATATAGCGCTAGCGAGCAGCCATAGTGACTGGCGATGCTGTGGAATGGAC  
GATATC  
CCGC  
AAGAGGCGCGGCGAGTACCGGCATAACCAAGCCTATGCTACAGCATCCAGGCTGACGGTGCCGAGGATGACG  
ATGAGCGCATTGTTAGATTTATACACGGTGCCTGACTGCGTTAGCAATTTAACTGTGATAAACTACCGCAT  
TAAAGCTTATCGATGATAAGCTGTCAACATGAGAATTC  
TTG

AAGACGAAAGGGCCTCGTGATACGCCTATTTTATAGGTTAATGTCATGATAATAATGTTTCTTAGACGTC  
\*MLU1-NOT1 fragment from pTHR219 containing the 6EGFs-Olinked into the MLU1-NOT1 sites of pTHR211 containing the Flori in the opposite orientation as pTHR219. This new plasmid is for in vitro mutagenesis of the Olinked and 6EGFs regions.



pTHR499

GAACCTTTAAAGTGTCTCATCATTGGAACACTTCTTCTGGGGCGAAACTCTCAAGGATC  
 TTACCGCTGTGAGATCCAGTTGATGTAACTCACTGTGACCCCAACTGATCTTCAGC  
 ATCTTTTACTTTACCAAGCGTTTCTGGGTGAGCAAAACAGGAGGCAAAATGCGGCAA  
 AAAAGGGAATAGGGGCGACACGGAAATGTTGATACTCATACTCTTCTTTTCAATAT  
 TATTGAGCATTATCAGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTA  
 GAAAAATAAACAAATAGGGGTTCCGCGACATTTCCCGGAAAGTGGCACCTGAGGTCT  
 AAGAAACCATTTATTATCATGACATTAACCTATAAAATAGGCGTATCAGGAGGCCCTTT  
 CGTCTTCAATTCAGGCGATGTTTACAGGTTATC  
 ATCGAT

\*XBA1-SAL1 fragment from pTHR499 containing the DNFL gene into the XBA1-SAL1 sites of pBES37 to make a mammalian expression plasmid that expresses DNFL using the MFSV promoter + CMV enhancer and has the IHR gene expressed off the SV40 late promoter. This plasmid also contains the HygromycinB gene.

pTHR515

AGCCGCGCAACAGAGCGAGAGGCAACTGATTGTTAGTTCAAATAAGGCAAGGTCATTTCAGGTCCTTGGGGCACC  
 CTGG  
 AACATCTGATGTTT  
 TCTAGA  
 CTGGAATTCGTGACGAGCTCCCTATAGTGAATCGTATTAGAG  
 GCGGACTTGGCCAAATTCATATCATGGTCAAGCTGTTTCTGTGTGAA  
 ATTGTTATCCGCTCACAATTCACACAACATACGAGCGGGAAGCATAAAG  
 TGTAAGSCCTGGGGTGCCTAATGAGTGAGCTAACTCATTAAATTGCTT  
 CGGCTCACTGCCCGCTTTCCAGTCCGGGAAACCTGTCTGCCAGCTGCATT  
 AATGAATCGGCCAACGCGCGGGGAG

\*CLAI-SMA1 fragment from pTHR515 containing the 3aa deletion of the Nterminus of DNFL into the CLAI-SMA1 partial of pTHR512.

pTHR515

CCCGGG  
 TAGAGCGCGAAGCAGTCTGTGCTCGACGCACTGG  
 CTGCCACCCGGCTGCGGCTCGG GGAAGACCGGCTGCCAGGSCCAGC  
 GCSCCAAGGACCGACCCCAAGCATGTTACCCAGGCGCGGagatctcgggcccg  
 atcgat  
 gccgcccgatatcgctcgagg  
 AAGCTT  
 GATATTCTATAGTGTACCTAAATAGCTTGGCTAATCATGGTCATA  
 GCTGTTTCTGTGTGAAATTGTTATCCGCTCACAATTCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGG  
 GTSCCTAATGAGTGAAGCTAACCACTTAATTTGCTTGGCTCACTGCCCGCTTTCCAGTCCGGGAAACCTGTCTGTGCCAG  
 CTGCATTAATGAATCGGCCAACGCGCGGGGAGGAGGCTTTGCTATTGGGCGCTCTTCCGCTTCTCGCTCACTGACTC  
 GCTGCGCTCGGTCGTTGCGCTGCGGCGAGCTGATCAGCTCACTCAAGGCGGTAATACG  
 \*COD2321 was used to delete the first 3 codons of the lectin domain and convert the fourth glycine of the signal to valine by in vitro mutagenesis in pTHR356. This created a STU1 site. Also, COD1690 was used to convert AMP sensitive to AMP resistant. This created a FET1 site.

pTHR356

TAGAGCGCGAAGCAGTCTGTGCTCGACGCACTGGCTGCCACCCGGCTGCGGCTCTGCG  
 GTGCGGGGAACCCAGGCGCCGCGGAGGCA  
 GCGGCG  
 CAAGGACCGAGACCCCAAGCATGTTACCCAG  
 GCGGCG  
 gagatctcgggcccgatcgatgccgcccgatcgctcgagg  
 AAGCTT  
 GATATTCTATAGTGTACCTAAATAGCTTGGCTAATCATGGTCATA

GCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAGAGTGTAAAGCCTGGG  
GTGCTTAATGAGTGAGCTAACCACATTAAATTTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCTGCCAG  
CTGCAATTAATGAATCGGCGCAACGCGCGGAGAGGCGGTTTCCGTATTGGGCGCTCTTCCGCTTCTCGCTCACTGACTC  
GCTGCGCTCGCTCTTGGGCTGCGGCGAGCGGTATCAGCTCACTCAAGGCGGTAATACG

\*HIND3-SMA1 fragment of pTHR322 containing the signal sequence of Thrombomodulin  
into the HIND3-SMA1 sites of pSELECT1. This plasmid will be used for mutagenesi  
s of the TM signal cleavage site.

AAGACGAAAGGGCCTCGTGATACGCCCTATTTTATAGGTTAATGTCATGATAATAATGTTTCTTAGACGTC  
\*MLU1-NOT1 fragment from pTHR127 containing the Met388-->Leu mutation in the 6E  
s into the MLU1-NOT1 sites of pTHR161 in order to make an E. coli expression ve  
tor that can be used for in vitro mutagenesis.

**pTHF:512**

\*NOT1-XBA1 fragment from pTHR498 containing the DNFL gene into the NOT1-XBA1 sites of pGEM9Zf-. This plasmid is designated 'A' in the strategy for construction of a Patent TM production plasmid.

pTHR211

AAGACGAAAGGGCCTCGTGATACGCCTATTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTC  
 \*SCA1-SAC1 fragment from pGEM3zf- containing the fl origin of replication into  
 the SCA1-SAC1 sites of pTHR161. This is a new mutagenesis vector that uses the c  
 posite strand as pTHR161.

## bTHR515

[illegible]

GCTGCGCTCGGTCGTTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACG  
 \*COD2321 was used to delete the first 3 codons of the lectin domain and convert the fourth glycine of the signal to valine by in vitro mutagenesis in pTHR356. This created a STU1 site. Also, COD1690 was used to convert AMP sensitive to A<sup>r</sup> resistant. This created a PST1 site.

## pTHR470

GAACTTTAAAGTGTCTCATCATTGSAAAACGTTCTTCGGGCGAAACTCTCAAGGATC  
 TTACCGCTGTTGAGATCCAGTTTCGATGTAACCCACTCGTGACCCCAACTGATCTTCAGC  
 ATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAA  
 AAAAGGGAATAAGGCGGACACGGAAATGTGAACTACTCACTCTTCCTTTTCAATAT  
 TATTGAAGCATTATTCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTA  
 SAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCT  
 AAGAAACCATATTATCATGACATTAACTATAAAAATAGGCGTATCACGAGGCCCTTT  
 CGTCTTCGAATTCAGGCCATGTTTGACAGCTTATC  
 ATCGAT

\*HIND3 fragment from pTHR359 containing the SV40 promoter into the HIND3 sites of pTHR359 to make an expression plasmid that expresses the DNFL gene off the SV40 early promoter and the DHFR gene off the SV40 late promoter. Note: The SV40 Late promoter initiates transcription from several locations. The main transcription initiation site is not included in this plasmid. Also Note: there are 3 ATGs between the late promoter and the ATG of DHFR which may hinder expression of DHFR. So expression may be too weak to express the DHFR gene.

5AAGCTTTAAAGTCTCATCATTTGAAAAAGCTTCTCGGGGCGAAAGCTCTGAGGATC  
 TTACGGCTCTTTGAGATCCAGTTGATSTAACTCACTGCTGCAACCACTGATCTTCAGC  
 ATCTTTTACTTTCAACCACTTTCTGGGTGAGCAAAAACAGGAGGGCAAAATGCCGCA  
 AAAAGGGAATAAGGGCGACACGGAATGTTGATACCTATCTCTCTCTTTTCAATAT  
 TATTGAAGCATTATACAGGTTATTCTCTCTGAGCAGATACATATTTGATATATTTA  
 GAAATATAAACAAATAGGGGTTCCGGGCACTTTCCCGGAAAGTGGCACTTGAAGCT  
 AGGAAACCATTTATTATCATGACATTTACCTATAAAATAGGGGTATCAGAGGCTCTT  
 CGTCTTCAATTCAGGCCATTTTGAAGCTTATC  
 ATCGAT

\*YBA1-NOT1 fragment from pTHR524 containing the 2aa deletion of the N-terminus plus the 4th aa Glu-Val, the 7aa deletion of the C-terminus, the S474A mutation, and the R456G, H457Q mutation of DNFL into the XBA1-NOT1 sites of pTHR493.

pTHR524  
 AGCGCGGAGCAGAGGCGAGAGCGAATGATGGTTAATTCAAATAGGCAAGGGTCAATTCAGGCTCTTGGGGCACC  
 CTGG  
 AACATCTGATGTTT  
 TCTAGA  
 CTGGAATTCCTGACGAGCTCCCTATAGTCTCTCTATTAGAG  
 GCTGACTTTGGCCAAATTCGTAACTCATGCTGTTTCTCTGTGAA  
 ATTGTTATCCGCTCACAATTCACACAACTACAGCCGGAAGCATAAAG  
 TGTAAAGCCTGGGGTGGCTAATGAGTGAATTAAGTCACTACATTAATTGCGTT  
 GCGCTCACTGCCCCGCTTTCCAGTCCGGGAAGCTGTGCTGCCAGCTGCATT  
 AATGAATCGGCCAAGCGCGGGGAG

\*MLU-NOT1 fragment from pTHR514 containing a 7aa deletion at the end of the oligo region of DNFL, the S474A, the R456G and the H457Q mutations into the MLU1-NOT1 sites of pTHR519.

THR514  
 AGCGAAGCGAGCAGGACTGGGGCGCGGCCAAAGCGGTGCGACAGTGTCCGAGAACGGGTGCGCATAGAAAT  
 TGCATCAACGCATATAGCGCTAGCAGCACGCCATAGTGAATGGCGATGCTGTGGAATGGAC  
 GATATC  
 CCSC  
 AAGAGGCCCGGCGAGTACCGGCATAACCAAGCTATGCTACAGCATCCAGGGTGACGGTGCCGAGGATGACG  
 ATGAGCGCATTTGTTAGATTTTCATACAGGCTGCTGACTGCGTTAGCAATTTAACTGTGATAAACTACCGCAT  
 TAAAGCTTATCGATGATAAGCTGTCAAACATGAGAATTC  
 TTG  
 AAGACGAAAGGGCCTCGTGATACGCTATTTTATAGGTTAATGTCATGATAAATGGTTTCTTAGACGTC  
 \*CDB2320 was used to delete 7 amino acids from the C-terminus of the O-link domain of TM by in vitro mutagenesis of pTHR511. This created an ACC1 site. Also, CDB1689 was used to convert AMP sensitive to AMP resistant. This created a PST1 site.

pTHR511  
 AGCGAAGCGAGCAGGACTGGGGCGCGGCCAAAGCGGTGCGACAGTGTCCGAGAACGGGTGCGCATAGAAAT  
 TGCATCAACGCATATAGCGCTAGCAGCACGCCATAGTGAATGGCGATGCTGTGGAATGGAC  
 GATATC  
 CCSC  
 AAGAGGCCCGGCGAGTACCGGCATAACCAAGCTATGCTACAGCATCCAGGGTGACGGTGCCGAGGATGACG  
 ATGAGCGCATTTGTTAGATTTTCATACAGGCTGCTGACTGCGTTAGCAATTTAACTGTGATAAACTACCGCAT  
 TAAAGCTTATCGATGATAAGCTGTCAAACATGAGAATTC  
 TTG  
 AAGACGAAAGGGCCTCGTGATACGCTATTTTATAGGTTAATGTCATGATAAATGGTTTCTTAGACGTC  
 \*MLU1-NOT1 fragment from pTHR493 containing the S474A, R456G and H457Q mutation of the DNFL gene into the MLU1-NOT1 sites of pTHR235.

pTHR525.seq

AAAAAGGCGG CTTCCTCTCC GGGGTGTGCG ACAAACCTCC CGGTACCTTC  
GAGTGCATCT GCGGGCCCGA CTCGCCCTTT GCTGGCCAGA TTGGCACCBA  
CTGTGACTCC GGCAGGTGG AGGTGSCGA CAGCGGCGCC GGCAGGCTCC  
CGCCGAGCCC GACGCGCGGG TCTACCTTGA CTCTCCGTG AGCGGCGGCT  
GCAGATCGAT GCGCGGCGGA TATCGCTCGA GGAAGCTTCC ATCCAGACAT  
GATAAGATAC ATTGATGAGT TTGGACAAAC CACAACAGAA ATGCAGTGAA  
AAAAATGCTT TATTGTGAA ATTTGTGATG CTATTGCTTT ATTTGTAACC  
ATTATAAGCT GCAATAAACG AETTAA

pTHR525.seqA

TTTCCAGGGTSCCCCAAGGACCTGAAATGACCTGTGCTTATTTGAACTAACDAATCAGTTGCTTCTCSC  
TTCTTTTCGCGGCTTCTGCTCCCGAGCTCAATAAAGAGCCDACAACCCCTCACTCGGGCGGCCAGTCTCTC  
CSATAGACTGCGTDCGCCCGGCTACCGAGCTCSAATTGATCTCAGGGAAGTGAAGAACAGAAAGTTAACTGGT  
AAGTTTAGTCTTTTTGTCTTTATTTGAGGTCCCGGATCGGAATTCCCATCGCCGCGGCATCGATCGGGCCCG  
AGATCTCGCGGCTTGGTAACTGCTTGGGCTCTGCTGCTTGGGCGCTGCGCCCTGCGAGGCTGCTTCTC  
CCCGAGCCCGAGCGCGGTGGCAGCCAGTGCCTCGAGCAGGACTGCTTGGGCTCTACCGGGCCCGCGAGCT  
TCCTCAATGCCAGTCAAGTCTGCGAGGACTGCGGGCCACCTAATGACAGTGCCTCTCGGTGCTGCGGA  
TGTCAATTCCTTGTACTGAAGCGCGAGCGGCTTNNNNNNNNNNNNNNCTGGATGGGCTGCGAGCTGCCA  
CCCGGCTGCGGCGAGCCCAAGCGCTCGGGCCCC

Table 11 shows the sequences of the various primers used to mutagenize the plasmids.

Table 11OLIGOMER SYNTHESIS REQUEST FORMCGD-2321

Oligomer size: 45

Project: TM

Purpose: convert fourth Gly of TM signal sequence to Val and delete AlaProAla of signal.

5'-----> 3'  
 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48  
 CTS CCA CCC GGC TGC GGC TCS GGG AAG ACC AGG CCT GCC AGG GGC AGC

Notes: Detect with STU1

Code Meaning

A Adenine  
 C Cytosine  
 G Guanine  
 T Thymine

OLIGOMER SYNTHESIS REQUEST FORMCGD-2320

Oligomer size: 51

Project: TM

Purpose: delete 7 amino acids from the 3' end of G-link region. by mutagenesis

5'-----> 3'  
 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51  
 CCC GAC GCC CGG GTC TAC CTT GAC TCC TCC GTG AGC GGC CGC CAG ATC CCC

Notes: Detect with ACC1

Code Meaning

A Adenine  
 C Cytosine  
 G Guanine  
 T Thymine

OLIGOMER SYNTHESIS REQUEST FORMCGD-2218

Oligomer size: 35

Project: tm

Purpose: mutagenic oligo to convert R456,H457 to G456,Q457 in DNFL. Screen with BAL1.

5'-----> 3'  
 3 6 9 12 15 18 21 24 27 30 33 35  
 SAC TCG GCC CTT GCT GGC CAG ATT GGC ACC GAC TG

Code Meaning  
A Adenine  
C Cytosine  
G Guanine  
T Thymine

OLIGOMER SYNTHESIS REQUEST FORM

COO-1886

Oligomer size: 27

Project: tm

Purpose: to destroy third potential gag site in cink domain by mutagenesis. This creates an NAR1 site.

5'-----> 3'

3 6 9 12 15 18 21 24 27  
GGC GAC AGC GGC GCC GGC GAG CCC CCG



**Example 10**      Separation and Purification of Clipped  
from Intact  $TM_{LEO}(CHO)M_{388}L$  by Affinity  
Chromatography

**A. Materials and Methods**

5      The clipped soluble human TM starting material was  
 $TM_{LEO}$  Peak A produced in CHO cells, lot #102491A. Based  
on N-terminal sequence analysis, this lot contains 10%  
two-chain form and 90% single-chain form. An immobilized  
10      anhydrotrypsin column (Pierce, Rockford, IL) was used to  
separate the clipped from unclipped  $TM_{LEO}$ . Anhydrotrypsin  
is a catalytically inert derivative of trypsin in which  
the active site serine residue 195 has been converted to  
a dehydroalanine residue. Under weakly acidic condi-  
15      tions, this column binds peptides that have a C-terminal  
Arg, Lys or S-aminoethyl-Cys residue. See Kumazaki, T.,  
et al. (1988) J. Biochem. 103:297.

Intact  $TM_{LEO}(CHO)M_{388}L$  contains a C-terminal Ser. The  
proteolytic cleavage at Arg<sub>456</sub> generates a second C-termi-  
nus ending in Arg. This dipeptide is covalently linked  
20      together by a disulfide bond. Thus, this column binds  
the two-chain clipped  $TM_{LEO}(CHO)M_{388}L$  present in the  
starting material and allows the single-chain intact  
 $TM_{LEO}(CHO)M_{388}L$  to flow through.

Approximately 1.29 ml of 0.87 mg/ml (459,000 U/ml;  
25      588,000 U total; 100%) starting material was diluted to  
15 ml using anhydrotrypsin binding buffer (0.05 M sodium  
acetate, pH 5.0, containing 20 mM calcium chloride and  
0.05% sodium azide) and loaded onto the column at 0.3  
ml/min using a syringe pump. While loading, the flow  
30      through was collected and contained intact  $TM_{LEO}(CHO)M_{388}L$ ,  
while the clipped  $TM_{LEO}(CHO)M_{388}L$  bound to the column. The  
column was then washed with 20 volumes of binding buffer  
and clipped  $TM_{LEO}(CHO)M_{388}L$  eluted using anhydrotrypsin  
elution buffer (0.1 M formic acid, pH 2.5). 21 fractions  
35      (1 min; ca. 300  $\mu$ l) were collected and diluted 1/1000 for  
an APC assay. The peak of activity was in fractions  
5-11. The pooled fractions comprised 2.1 ml and  
contained 45,771 U/ml (96,000 U total, 16%). Aliquots of

the starting material, flow through and the pooled eluate were desalted into 0.2% NEM, pH 7.5 for analysis.

The  $K_d$  for soluble human TM binding to human thrombin was determined in microtiter plates. Soluble TM samples were diluted (all dilutions are into APC assay diluent) to a working solution of approximately 3 nM; human thrombin was diluted from a stock solution to working dilutions of 600, 300, 150, 75, 37.5, 18.8, 9.4, 4.7, 2.3, 1.2, 0.6 and 0 nM. Working solutions of additional reagents were: human protein C, 1.5  $\mu$ M; hirudin, 160 U/ml; and S-2266 substrate 1 mM. Quadruplicate 25  $\mu$ l samples of each thrombin dilution were added to one-half column of the microtiter plate; 25  $\mu$ l aliquots of soluble TM are added to three of four test rows and 25  $\mu$ l APC assay diluent replaces TM in one test row. The plate is mixed by tapping gently, sealed with a plate sealer and incubated at room temperature for 5 min. 25  $\mu$ l of protein C was added to each well, the plate was tapped to mix, sealed and incubated 15 min at room temperature; then 25  $\mu$ l of hirudin was added to each well and the plate was tapped to mix. 100  $\mu$ l of S-2266 was added to each well, the plate was tapped to mix and read kinetically for 15 min in a Molecular Devices Plate Reader (Menlo Park, CA) using the SoftMax analysis and control software program. Linear rates of S-2266 hydrolysis were used as a measure of activated protein C formed after subtracting the appropriate background rates. This data was fit to give a  $K_d$  using the program Enzfitter, Robin Leatherbarrow, Elsevier-BioSoft, Cambridge, UK on an IBM PC.

#### B. Results

The following analyses were done on the starting material, flow through and the pool: 1) SDS-PAGE under reducing conditions (Figs. 6 and 7), 2) APC assay (two dilutions in triplicate), 3) amino acid analysis in duplicate, 4) N-terminal sequence analysis, and 5) determination of  $K_d$  for human  $\alpha$ -thrombin (results shown in Tables 12 and 13).

TABLE 12Comparison of Clipped-TM<sub>LEO</sub>(CHO)M388L and Intact-TM<sub>LEO</sub>(CHO)M388L

TM <sub>LEO</sub> Form	Amino Acid Analysis (mg/ml)	APC Activity (U/ml)	Specific Activity (kU/mg)	Thrombin K <sub>D</sub> (nM)
Lot #102491 (10% clipped)	0.048±0.01	23,900±570	498±100	9.9±1.1
Intact-TM <sub>LEO</sub> (flow through)	0.035±0.007	19,400±350	554±110	9.6±0.9
Clipped-TM <sub>LEO</sub> (bound pool)	0.026 ±0.003	9,030±150	347±40	19±1

TABLE 13N-terminal Sequence Analysis of TM<sub>LEO</sub>(CHO)M388L Lot #102491A (Starting Material); Clipped-TM<sub>LEO</sub>(CHO)M388L (Pool); and Intact-TM<sub>LEO</sub>(CHO)M388L (Flow Through)

Sequence	Lot #102491A	Flow Through (Intact)	Pool (Clipped)
		pmol (% of total)	
1) HIGTDCDSGK... (clip site)	3 (8%)	nd <sup>1</sup>	186 (53%)
2) FPAPAEPQPG... (N-terminus #1)	14 (36%)	30 (32%)	40 (11%)
3) APAEPQPGGS... (N-terminus #2)	22 (56%)	65 (68%)	127 (36%)

<sup>1</sup> nd = Not detectable.

These results show that TM<sub>LEO</sub>(CHO)M<sub>388</sub>L can be cleanly separated into a form which is clipped after R<sub>456</sub> and an intact form by an affinity chromatographic purification step. Clipped TM<sub>LEO</sub>(CHO)M<sub>388</sub>L was shown to have 63 ± 15% of the specific activity of intact TM<sub>LEO</sub>(CHO)M<sub>388</sub>L. The two forms were compared in an assay in which the activation of protein C is measured in increasing concentrations of human thrombin and it was determined that clipped

$TM_{LEO}(CHO)M_{388}L$  binds thrombin two times less tightly than intact  $TM_{LEO}(CHO)M_{388}L$ .

5. The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

10. From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

WHAT IS CLAIMED IS:

1. A thrombomodulin protein analog, wherein the amino acid sequence of a protease cleavage site is modified, whereby the analog is resistant to protease cleavage at that site.

2. A thrombomodulin analog of claim 1, wherein the amino acid sequence of the unmodified protease cleavage site of the protein is between Arg<sup>456</sup> and His<sup>457</sup> of natural thrombomodulin.

3. A thrombomodulin analog of claim 2, wherein the amino acid sequence of the protease cleavage site has been modified to Gly<sup>456</sup>-Gln<sup>457</sup>.

4. A thrombomodulin protein analog, wherein the amino acid sequence of the N-terminus is modified, whereby the analog has a single N-terminus.

5. A thrombomodulin of claim 4, wherein the N-terminal amino acid is Glu<sup>4</sup> of natural thrombomodulin.

6. A thrombomodulin protein analog, wherein the amino acid sequence of the C-terminus is modified, whereby the analog has a single C-terminus.

7. A thrombomodulin of claim 6, wherein the C-terminal amino acids are -Pro<sup>489</sup>-Pro<sup>490</sup> of natural thrombomodulin.

8. A process of enhancing the activity of thrombomodulin, wherein the thrombomodulin comprises a mixture of single- and two-chain thrombomodulin polypeptides, comprising removing therefrom two-chain thrombomodulin.

9. A process of enhancing the activity of thrombomodulin, comprising preventing the cleavage of single-chain thrombomodulin.

10. A DNA sequence coding for a thrombomodulin analog of claim 1.

11. A DNA sequence coding for a thrombomodulin analog of claim 4.

12. A DNA sequence coding for a thrombomodulin analog of claim 6.

13. A vector comprising a DNA sequence of claim 10, operably linked to a promoter sequence capable of being expressed in a suitable host.

14. A vector comprising a DNA sequence of claim 11 operably linked to a promoter sequence capable of being expressed in a suitable host.

15. A vector comprising a DNA sequence of claim 12, operably linked to a promoter sequence capable of being expressed in a suitable host.

16. A vector of claim 13 capable of being expressed in a CHO cell.

17. A vector of claim 14, capable of being expressed in a CHO cell.

18. A vector of claim 15, capable of being expressed in a CHO cell.

19. A pharmaceutical composition comprising an effective amount of a thrombomodulin analog of claim 1 and a pharmaceutically acceptable excipient.

20. A method of treating thrombotic disease, comprising administering an effective amount of a thrombomodulin analog of claim 1.

21. A method of expressing a single-chain polypeptide in a cell which produces a protease capable of cleaving said polypeptide, comprising:

a) detecting the presence of cleaved polypeptide in the expression product;

b) determining the cleavage site by amino acid sequence analysis;

c) modifying the nucleotide sequence of the gene which codes for the polypeptide at the cleavage site, whereby the thus-produced polypeptide is resistant to protease cleavage at said site and maintains biological activity;

d) expressing the modified gene in a cell which produces the protease; and

e) isolating the thus-produced single-chain polypeptide.

22. A method of claim 21, wherein the cleaved polypeptide lacks biological activity and/or is a competitive inhibitor of the uncleaved polypeptide.

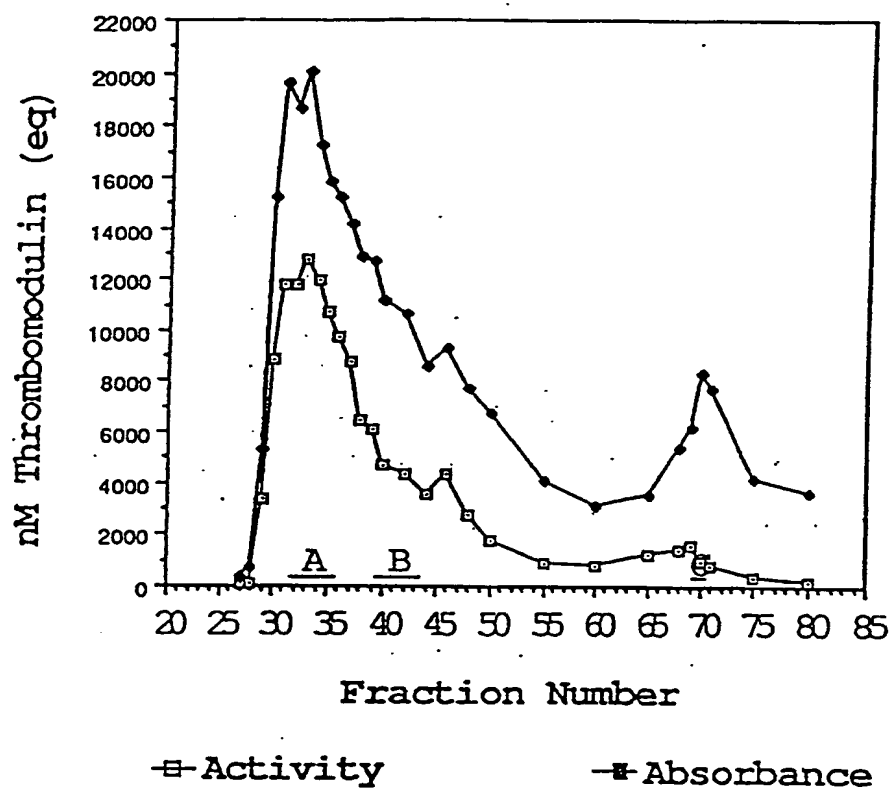
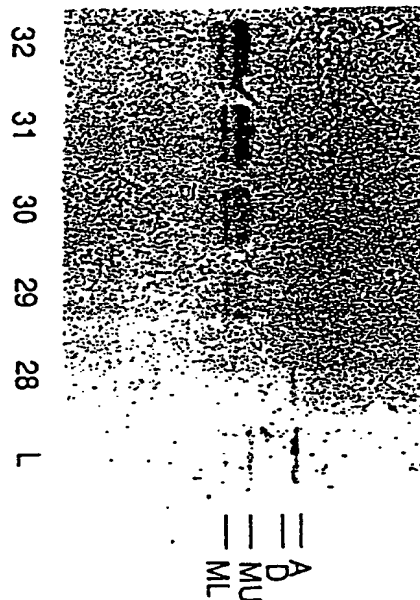


FIGURE 1

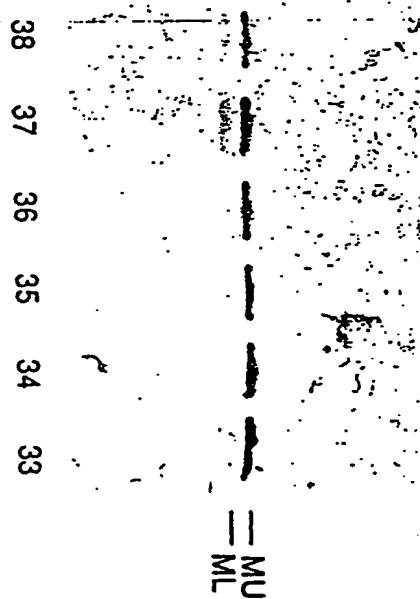


2/7

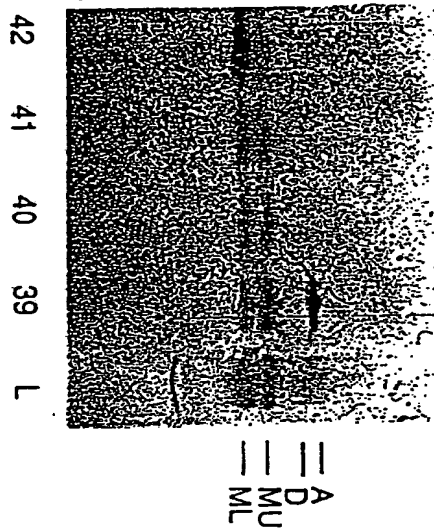
A.



B.



C.



D.

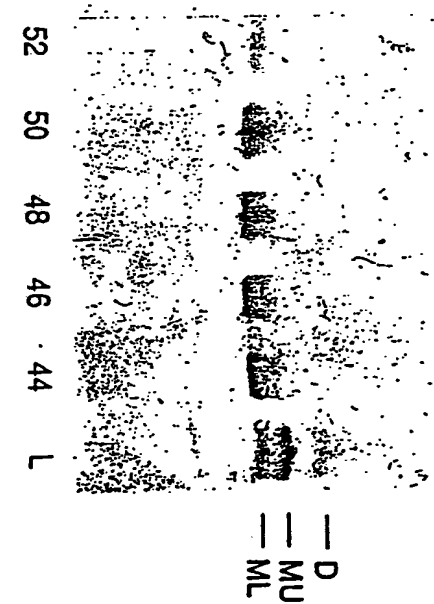


FIGURE 2

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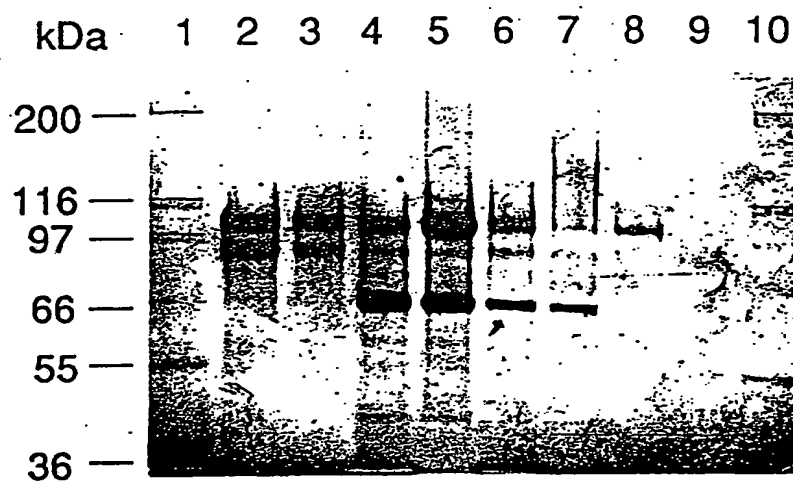


FIGURE 3

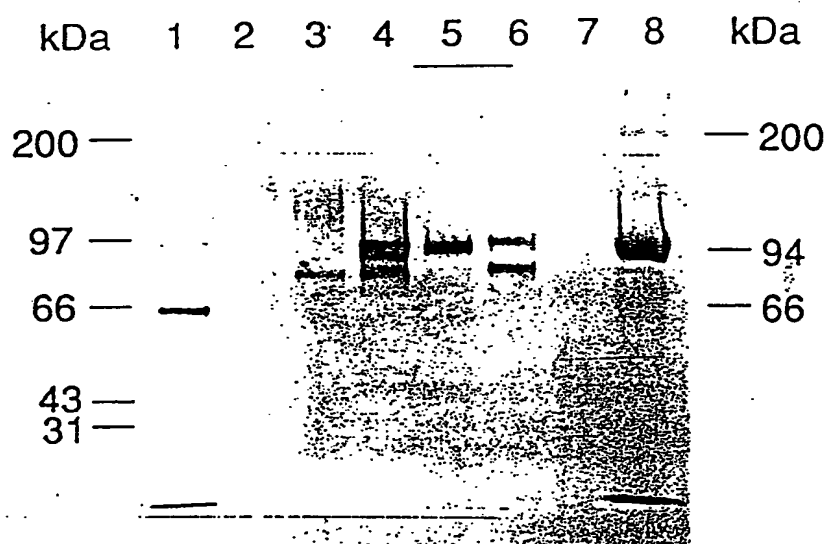
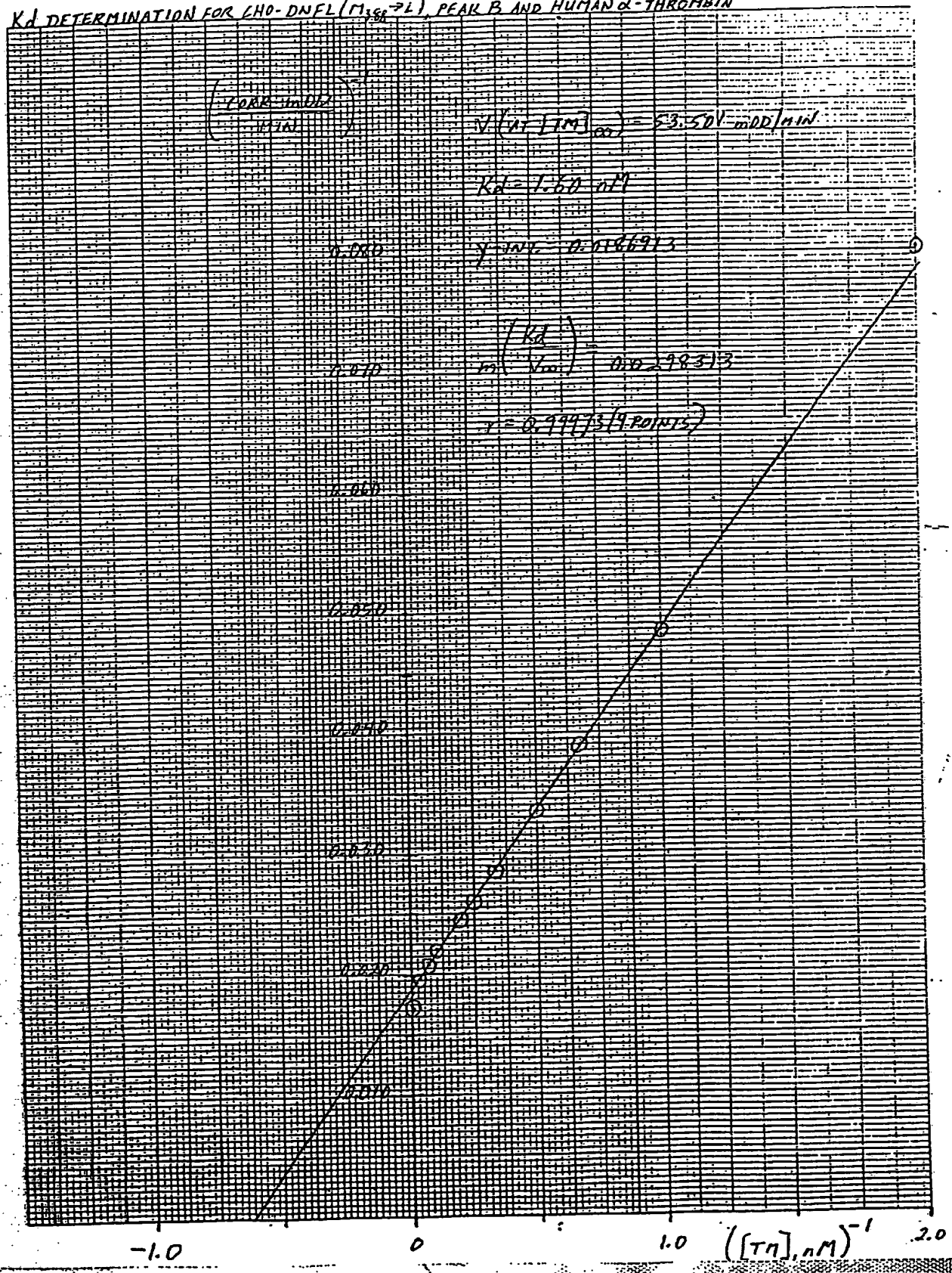


FIGURE 4

$K_d$  DETERMINATION FOR CHO-DNFL ( $M_{156} \rightarrow L$ ) PEAK B AND HUMAN  $\alpha$ -THROMBIN



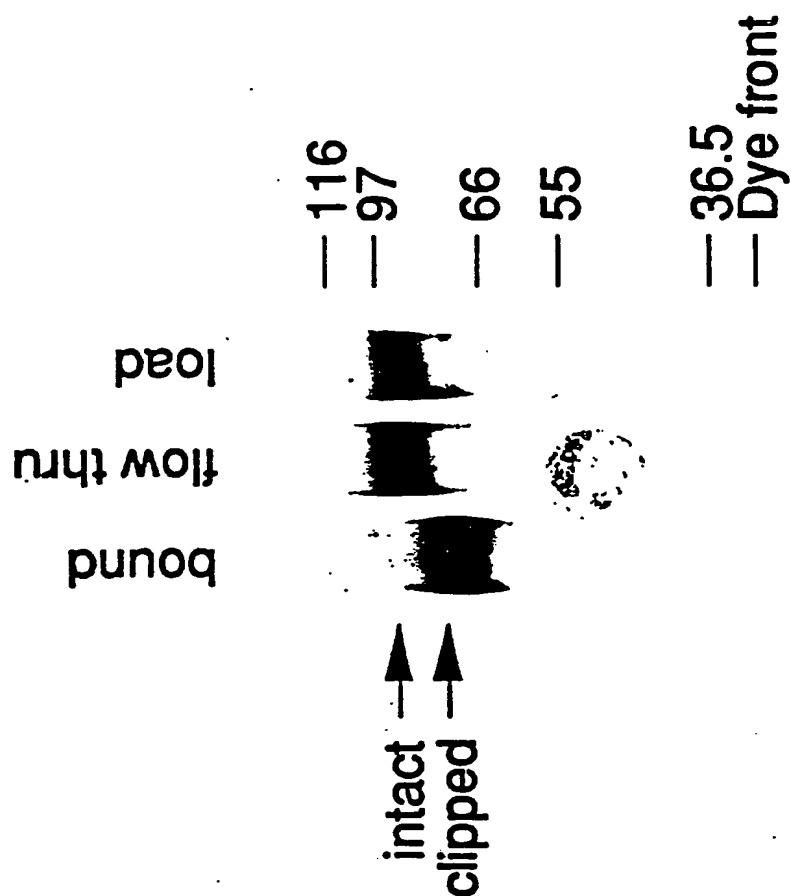


Figure 6

Gel Scan of Clipped TM-LEO PKA from CHO Cells  
Fractionated on Anhydrotrypsin Column

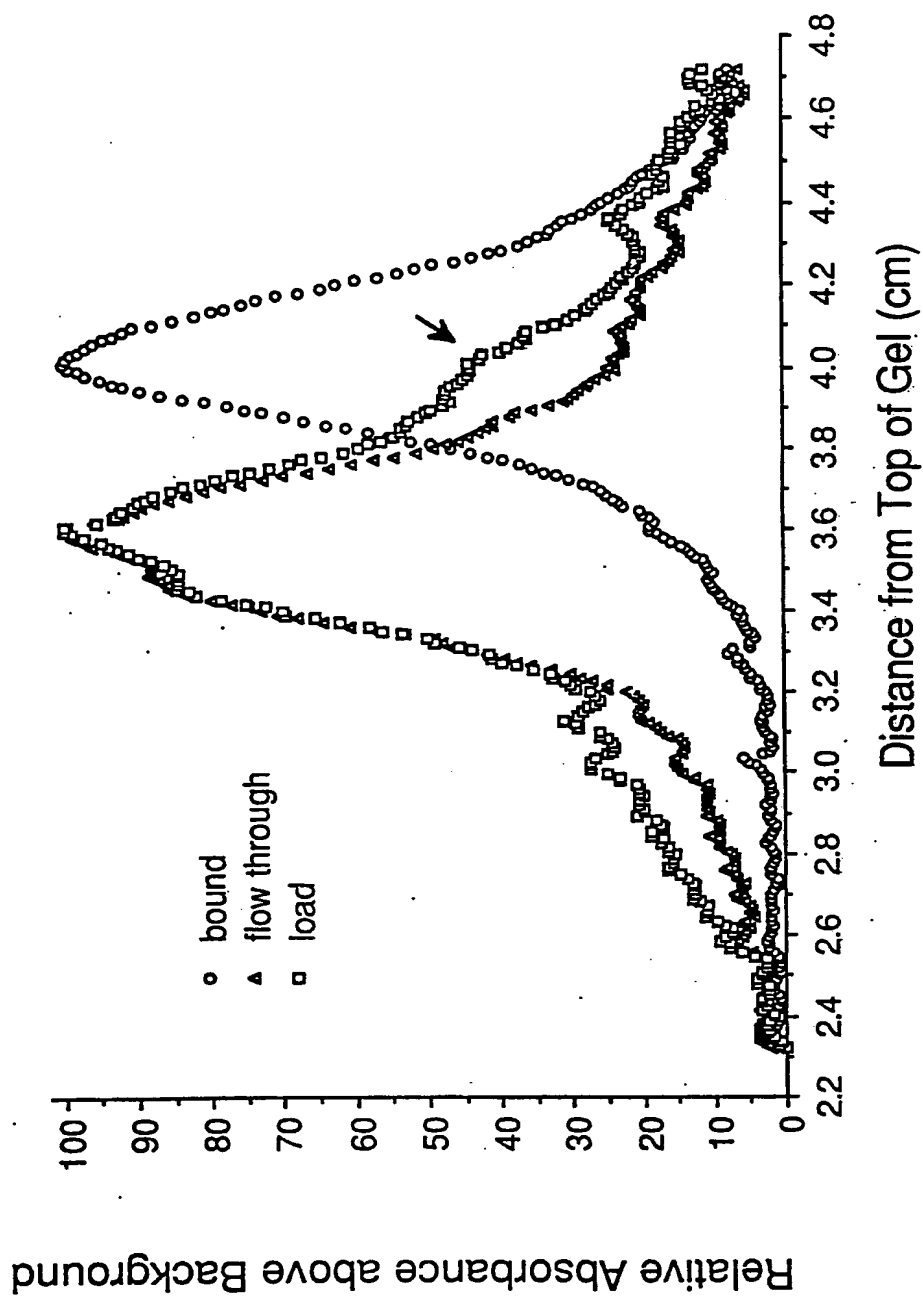


Figure 7

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/01120

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : A61K 37/02; C07H 21/04; C07K 13/00; C12N 15/12, 15/63;  
US CL : 435/320.1; 514/2; 530/350; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2, 320.1; 514/2; 530/350, 381; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	Journal of Biological Chemistry, Volume 268, No. 4, issued 05 February, 1993, N. Nagashima <i>et al.</i> , "Alanine-scanning Mutagenesis of the Epidermal Growth Factor-like Domains of Human Thrombomodulin Identifies Critical Residues for its Cofactor Activity", pages 2888-2992, entire document.	1-20
X	Journal of Biological Chemistry, Volume 261, No. 21., issued 25 July 1990, Parkinson <i>et al.</i> , "Stable Expression of a Secretable Deletion Mutant of Recombinant Human Thrombomodulin in Mammalian Cells", pages 12602-12610.	6, 12, 14, 17,
X	WO,A, 88/09811, (B. Nexø <i>et al.</i> ) 15 December 1988, entire document, especially claims 1-5, 7, 17, 19-23, 31-33, 36-37	1, 2, 3, 6, 10, 12-13, 15-16, 18-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	&	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

06 May 1993

Date of mailing of the international search report

17 MAY 1993

Name and mailing address of the ISA/US  
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Authorized officer

GABRIELE E. BUGAISKY

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

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## INTERNATIONAL SEARCH REPORT

International application No.  
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A, 90/10081 (Morser <u>et al.</u> ) 07 September 1990, entire document, especially pages 25-27, 54-55	4, 6, 11, 12, 14, 15, 17-20



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/01120

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:  
1-20
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)•

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/01120

### B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Dialog files 155, 5, 73, 357 (Medline, BioSis, Embase, Biotechnology Abstracts),  
Search terms: thrombomodulin, mutat?, mutant??, cleav?, process, matur?, DNA, single chain

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- I. Claims 1-9 and 19-20, drawn to thrombomodulin analogs and methods of making such, classified in Class 530, subclass 350.
- II. Claims 10-18, drawn to recombinant DNA compositions, classified in class 435, subclass 320.1.
- III. Claims 21-22, drawn to a method of use of DNA, classified in Class 435, subclass 69.1.

Groups I and II are drawn to different chemical compositions; the existence of either one is not dependent on the existence of the other and each can be chemically synthesized. Group III is a method of use of the second invention.

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